

NOVEL COMPOSITIONS WITH CHITINASE ACTIVITY

Cross-References to Related Applications

[0001] This application is a continuation-in-part of and claims priority to and benefit of U.S. Application Serial No. 10/389,432 filed on March 14, 2003, which is a continuation of and claims priority to and benefit of U.S. Application Serial No. 10/290,086 filed on November 6, 2002, which claims priority to and benefit of U.S. Provisional Patent Application Serial No. 60/337,029, filed on November 7, 2001 and U.S. Provisional Patent Application Serial No. 60/420,666, filed on October 22, 2002. The disclosures of these applications are incorporated herein by reference in their entireties for all purposes.

Background of the Invention

[0002] Plant chitinases belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack (Giazinazzi, *In Plant Microbe Interactions, Molecular and Genetic Perspectives*, Vol. 1 (ed. Nester, E. W. & Kosuge T., 1987) 321-342; Boller, T., *Id.*, Vol. 2, 385-413; Legrand, M., *et al. Proc. Natl. Acad. Sci USA* 84:6750-6754 (1987); Collinge, *et al.*, *Plant J.* 3:31-40 (1993). Chitinases catalyze the hydrolysis of the β -1,4 linked N-acetylglucosamine polymers that form chitin chains, a major component of fungal cell walls. Chitinases have been divided based on their structure into at least four classes (classes i-iv). *See, e.g.*, Hamel, *et al.*, *J. Mol. Evol.* 44(6):614-24 (1997).

[0003] Even though chitinases have been shown to inhibit the hyphal tip growth of many fungi *in vitro* (Mauch, *et al. Plant Physiol.* 88:936-942 (1988)), a plant's natural defense mechanisms are often insufficient to prevent an invasion by the pathogen (Neuhaus, *et al. Plant Mol. Biol.* 16, 141-151 (1991)). The consequences of plant disease caused by fungal pathogens can be significant losses in crop quality and yields. Plants over-expressing chitinases under the control of a strong constitutive promoter have been engineered and have shown improved resistance against fungal pathogens under laboratory conditions (Broglie, *et al. Science* 254:1194-1197 (1991), Vierheilig, *et al. Molecular Plant-microbe Interactions* 6:261-264 (1993); Asao, *et al. Plant Biotech.* 14: 145-149 (1997); Tabei, *Plant Cell Rep.* 17: 159-164 (1998); Lorito *et al.*, *Proc. Natl. Acad. Sci. USA* 95:7860-7865 (1998). Further, plants constitutively over-expressing a hybrid endochitinase exhibited improved tolerance to fungal diseases in field tests (Grisson, *et al. Nature Biotech.* 14:643-646 (1996)).

[0004] The expression of chitinases in plants is therefore useful to enhance resistance in plants to fungi, including fungal pathogens. Chitinases expressed in plants are also reported to have anti-insect activity. *See, e.g., Ding, et al., Transgenic Res. 7(2):77-84 (1998).* Additionally, chitinases are useful in industrial processes aimed at the bioconversion of shellfish chitin waste (Cosio, *et al. J. Food Sci.* 47:901-905 (1982)).

[0005] In addition, nematode infection is a significant problem in the farming of many agriculturally significant crops. For example, soybean cyst nematode (*Heterodera glycines*, herein referred to as “SCN”) is a widespread pest that causes substantial damage to soybeans every year. Such damage is the result of the stunting of the soybean plant caused by the cyst nematode. The stunted plants have smaller root systems, show symptoms of mineral deficiencies in their leaves, and wilt easily. The soybean cyst nematode is believed to be responsible for yield losses in soybeans that are estimated to be in excess of \$500 million per year. Other pathogenic nematodes of significance to agriculture include the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida*, which are key pests of the potato, while the beet cyst nematode *Heterodera schachtii* is a major problem for sugar beet growers in Europe and the United States.

[0006] Although expression of chitinases can be useful to reduce infection by fungal pathogens and other pests, constitutive overexpression of foreign proteins in crop plants has a potentially yield-reducing metabolic cost. Moreover, it is commonly found that particular chitinases only have antifungal activity against a narrow range of fungal pathogens. Further, it is known that certain nematodes, such as the soybean cyst nematode (“SCN”), can inhibit certain plant gene expression at the nematode feeding site. Thus, in implementing a transgenic approach to pathogen control, an important factor is to increase the expression of desirable genes in response to pathogen attack. Thus, chitinases with high activity and broad specificity are needed.

Summary of the Invention

[0007] Compositions and methods involved in pathogen defense for enhancing nematode and fungal resistance in plants are provided. The compositions include nucleic acid molecules comprising a sequence useful in pathogen control. The invention further includes expression constructs comprising nucleic acid sequences, operably linked to regulatory promoters, the nucleic acid sequences encoding proteins useful in pathogen control of the invention or other combinations of these novel sequences of the invention with other nucleotide sequences, as well as vectors and transformed plant cells, plants and seeds comprising these constructs. The

pathogen control sequences include novel proteins involved in enhancing a plants resistance to fungal and nematode pathogens. These proteins, and the nucleotide sequences encoding them, provide an opportunity for engineered plants with improved resistance to fungal and nematode plant pathogens. Amino acid sequences of these proteins are provided as well as purified proteins themselves. Polynucleotides having nucleic acid sequences encoding these polypeptides are also provided. The DNA sequences encoding these proteins can be used to transform plants, bacteria, fungi, yeasts, and other organisms for the control of pests.

[0008] In addition, the invention encompasses fragments and variants of the particular sequences as defined herein. In another aspect of the present invention, expression cassettes and transformation vectors comprising the isolated nucleotide sequences are disclosed. The transformation vectors can be used to transform plants and express the pathogen control genes in the transformed cells. In this manner, the pathogen resistance, particularly fungal and nematode resistance, of plants can be improved. Transformed cells as well as regenerated transgenic plants and seeds containing and expressing the isolated DNA sequences and protein products are also provided.

Brief Description of the Drawings

[0009] Figure 1 illustrates the inhibition of *Fusarium moniliforme* hyphal growth in the presence of purified chitinases (chitinase A (SEQ ID NO: 1) and r2C2 (SEQ ID NO: 12)).

[0010] Figure 2 illustrates a comparative nucleotide alignment between the gene sequence encoding chitinase A (SEQ ID NO: 1) and a selection of the polynucleotides of the invention (r1B6 (SEQ ID NO: 3), r1B10 (SEQ ID NO: 5), r1D4 (SEQ ID NO: 7), r2A2 (SEQ ID NO: 9); r2C2 (SEQ ID NO: 11), r2E1 (SEQ ID NO: 13), and r2H2 (SEQ ID NO: 15)).

[0011] Figure 3 illustrates a comparative amino acid alignment between chitinase A (SEQ ID NO: 1) and a selection of the gene products of the invention (r1B6 (SEQ ID NO: 4), r1B10 (SEQ ID NO: 6), r1D4 (SEQ ID NO: 8), r2A2 (SEQ ID NO: 10), r2C2 (SEQ ID NO: 12), r2E1 (SEQ ID NO: 14), and r2H2 (SEQ ID NO: 16)).

[0012] Figures 4A and 4B illustrate the antifungal activity of some identified chitinases. Purified chitinases were added to germinating spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. In Figure 4A, the average concentration of chitinase required to inhibit fungal growth by 50% is reported. In Figure 4B, improvement of clones 4M1/1_H2 (SEQ ID NO: 24),

4M1/11_C10 (SEQ ID NO: 22), and 4M1/26_C2 (SEQ ID NO: 26) as compared to “hit” r2C2 (SEQ ID NO: 12) is reported.

[0013] Figure 5 illustrates antifungal activity of variant 4M1/11_C10 (SEQ ID NO: 22), as compared to the wild-type protein chitinase A (SEQ ID NO: 1) (labeled “1-2SCH” in the figure) and variant “r2C2” (SEQ ID NO: 12).

[0014] Figure 6 illustrates a comparative nucleotide alignment between a selection of the polynucleotides of the invention (4M1/1_H2 (SEQ ID NO: 23), 4M1/11_C10 (SEQ ID NO: 21), and 4M1/26_C2 (SEQ ID NO: 25)).

[0015] Figure 7 illustrates a comparative amino acid alignment between a selection of the gene products of the invention (4M1/1_H2 (SEQ ID NO: 24), 4M1/11_C10 (SEQ ID NO: 22), and 4M1/26_C2 (SEQ ID NO: 26)).

[0016] Figure 8 illustrates antifungal activity of chitinases on solid medium. Purified chitinases were incorporated into cooling agar-VMM medium in 96-well microtiter plates. One hundred *Fusarium moniliforme* spores were added to each well and pictures were taken at 40h, 48h, and 58h after the spore addition, on an inverted microscope (4x objective). Hyphal growth is compared in a control well not containing chitinase and in wells containing the wild-type chitinase 1-2SCH (chitinase A) (SEQ ID NO: 1) and the hit 4N1/88 F9 (SEQ ID NO: 56).

[0017] Figure 9 illustrates antifungal activity of improved chitinases (r1AB2 (SEQ ID NO: 28), r1AD4 (SEQ ID NO: 30), r1AD6 (SEQ ID NO: 32), r1AG9 (SEQ ID NO: 34), r1AH8 (SEQ ID NO: 36), r1AH9 (SEQ ID NO: 38), r1BG5 (SEQ ID NO: 40), r2C5 (SEQ ID NO: 42)). Purified chitinases were added to germinating spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. The average concentration of chitinase required to inhibit fungal growth by 50% is reported. The horizontal line at IC₅₀=60 µg/ml corresponds to the activity of hit r2C2 (SEQ ID NO: 12). Protein concentrations were determined with ovalbumin as a standard, therefore, IC₅₀ values are in ovalbumin equivalents.

[0018] Figure 10 illustrates improvements in the antifungal activity of later shuffled chitinases over previously shuffled chitinases (r1AB2 (SEQ ID NO: 28), r1AD4 (SEQ ID NO: 30), r1AD6 (SEQ ID NO: 32), r1AG9 (SEQ ID NO: 34), r1AH8 (SEQ ID NO: 36), r1AH9 (SEQ ID NO: 38), r1BG5 (SEQ ID NO: 40), r2C2 (SEQ ID NO: 12), r2C5 (SEQ ID NO: 42), 4M1/1_H2 (SEQ ID NO: 24), 4M1/11_C10 (SEQ ID NO: 22), 4M1/26_C2 (SEQ ID NO: 26), 4N1/2_H9 (SEQ ID NO: 50), 4N1/11_B11 (SEQ ID NO: 62), 4N1/14_B3 (SEQ ID NO: 58), 4N1/23_G4 (SEQ ID NO: 52), 4N1/30_D3 (SEQ ID NO: 64), 4N1/33_F4 (SEQ ID NO: 60),

4N1/35_G5 (SEQ ID NO: 66), 4N1/68_E4 (SEQ ID NO: 54), 4N1/75_D3 (SEQ ID NO: 48), 4N1/80_F8 (SEQ ID NO: 46), 4N1/88_F9 (SEQ ID NO: 56), and 4N1/95_H3 (SEQ ID NO: 44)).

Purified chitinases were added to germinating spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. Activity measurements are expressed in number of folds the clones are improved over the wild-type clone 1-2SC (chitinase A) (SEQ ID NO: 1).

[0019] Figures 11A to 11C are a comparative nucleotide alignments between a selection of the polynucleotides of the invention (r1AB2 (SEQ ID NO: 27), r1AD4 (SEQ ID NO: 29), r1AD6 (SEQ ID NO: 31), r1AG9 (SEQ ID NO: 33), r1AH8 (SEQ ID NO: 35), r1AH9 (SEQ ID NO: 37), r1BG5 (SEQ ID NO: 39), r2C5 (SEQ ID NO: 41), 4N1/95_H3 (SEQ ID NO: 43), 4N1/80_F8 (SEQ ID NO: 45), 4N1/75_D3 (SEQ ID NO: 47), 4N1/2_H9 (SEQ ID NO: 49), 4N1/23_G4 (SEQ ID NO: 51), 4N1/68_E4 (SEQ ID NO: 53), 4N1/88_F9 (SEQ ID NO: 55), 4N1/14_B3 (SEQ ID NO: 57), 4N1/33_F4 (SEQ ID NO: 59), 4N1/11_B11 (SEQ ID NO: 61), 4N1/30_D3 (SEQ ID NO: 63), and 4N1/35_G5 (SEQ ID NO: 65)).

[0020] Figure 12 illustrates a comparative amino acid alignment between a selection of the polypeptides of the invention (r1AB2 (SEQ ID NO: 28), r1AD4 (SEQ ID NO: 30), r1AD6 (SEQ ID NO: 32), r1AG9 (SEQ ID NO: 34), r1AH8 (SEQ ID NO: 36), r1AH9 (SEQ ID NO: 38), r1BG5 (SEQ ID NO: 40), r2C5 (SEQ ID NO: 42), 4N1/95_H3 (SEQ ID NO: 44), 4N1/80_F8 (SEQ ID NO: 46), 4N1/75_D3 (SEQ ID NO: 48), 4N1/2_H9 (SEQ ID NO: 50), 4N1/23_G4 (SEQ ID NO: 52), 4N1/68_E4 (SEQ ID NO: 54), 4N1/88_F9 (SEQ ID NO: 56), 4N1/14_B3 (SEQ ID NO: 58), 4N1/33_F4 (SEQ ID NO: 60), 4N1/11_B11 (SEQ ID NO: 62), 4N1/30_D3 (SEQ ID NO: 64), and 4N1/35_G5 (SEQ ID NO: 66)).

[0021] Figure 13 illustrates the antifungal activity of improved chitinases from the 4th round of shuffling (4Q2/10_B8 (SEQ ID NO: 76) and 4Q2/13_F8 (SEQ ID NO: 78)). Purified chitinases were added to germinating spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. The average concentration of chitinase required to inhibit fungal growth by 50% (IC₅₀) is reported. IC₅₀ values are given in bovine serum albumin (BSA) and in ovalbumin equivalents, thus reflecting the standard used for the determination of protein concentrations.

[0022] Figure 14 illustrates the antifungal activity of improved chitinases from the 4th round of shuffling (4P1/2_B5 (SEQ ID NO: 68), 4P1/7_H9 (SEQ ID NO: 70), Q1/3_H10 (SEQ ID NO: 72), 4Q1/5_B11 (SEQ ID NO: 74), 4R2/1_G10 (SEQ ID NO: 80), 4R2/5_H11 (SEQ ID NO: 82), and 4R2/9_B2 (SEQ ID NO: 84)). Purified chitinases were added to germinating

spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. The average concentration of chitinase required to inhibit fungal growth by 50% (IC₅₀) is reported. IC₅₀ values are given in bovine serum albumin (BSA) and in ovalbumin equivalents, thus reflecting the standard used for the determination of protein concentrations.

[0023] Figure 15 illustrates improvements in the antifungal activity of later shuffled chitinases over previously shuffled chitinases (r1AB2 (SEQ ID NO: 28), r1AD4 (SEQ ID NO: 30), r1AD6 (SEQ ID NO: 32), r1AG9 (SEQ ID NO: 34), r1AH8 (SEQ ID NO: 36), r1AH9 (SEQ ID NO: 38), r1BG5 (SEQ ID NO: 40), r2C2 (SEQ ID NO: 12), r2C5 (SEQ ID NO: 42), 4M1/1_H2 (SEQ ID NO: 24), 4M1/11_C10 (SEQ ID NO: 22), 4M1/26_C2 (SEQ ID NO: 26), 4N1/2_H9 (SEQ ID NO: 50), 4N1/11_B11 (SEQ ID NO: 62), 4N1/14_B3 (SEQ ID NO: 58), 4N1/23_G4 (SEQ ID NO: 52), 4N1/30_D3 (SEQ ID NO: 64), 4N1/33_F4 (SEQ ID NO: 60), 4N1/35_G5 (SEQ ID NO: 66), 4N1/68_E4 (SEQ ID NO: 54), 4N1/75_D3 (SEQ ID NO: 48), 4N1/80_F8 (SEQ ID NO: 46), 4N1/88_F9 (SEQ ID NO: 56), 4N1/95_H3 (SEQ ID NO: 44), 4P1/2_B5 (SEQ ID NO: 68), 4P1/7_H9 (SEQ ID NO: 70), Q1/3_H10 (SEQ ID NO: 72), 4Q1/5_B11 (SEQ ID NO: 74), 4R2/1_G10 (SEQ ID NO: 80), 4R2/5_H11 (SEQ ID NO: 82), 4R2/9_B2 (SEQ ID NO: 84), 4Q2/10_B8 (SEQ ID NO: 76) and 4Q2/13_F8 (SEQ ID NO: 78)). Purified chitinases were added to germinating spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. Activity measurements are expressed in number of folds the clones are improved over the wild-type clone 1-2SC (chitinase A) (SEQ ID NO: 1).

[0024] Figure 16 a comparative nucleotide alignment between the sequences 4Q2/10_B8 (SEQ ID NO: 76) and 4Q2/13_F8 (SEQ ID NO: 78).

[0025] Figure 17 illustrates a comparative amino acid alignment between 4Q2/10_B8 (SEQ ID NO: 76) and 4Q2/13_F8 (SEQ ID NO: 78) gene products of the invention.

[0026] Figures 18A and 18B are comparative nucleotide alignments between the sequences shuffling 4P1/2_B5 (SEQ ID NO: 68), 4P1/7_H9 (SEQ ID NO: 70), Q1/3_H10 (SEQ ID NO: 72), 4Q1/5_B11 (SEQ ID NO: 74), 4R2/1_G10 (SEQ ID NO: 80), 4R2/5_H11 (SEQ ID NO: 82), and 4R2/9_B2 (SEQ ID NO: 84).

[0027] Figure 19 illustrates a comparative amino acid alignment between shuffling 4P1/2_B5 (SEQ ID NO: 68), 4P1/7_H9 (SEQ ID NO: 70), Q1/3_H10 (SEQ ID NO: 72), 4Q1/5_B11 (SEQ ID NO: 74), 4R2/1_G10 (SEQ ID NO: 80), 4R2/5_H11 (SEQ ID NO: 82), and 4R2/9_B2 (SEQ ID NO: 84) gene products of the invention.

Detailed Description

[0028] For purposes of the present invention, “anti-fungal activity” refers to the ability of a polypeptide to inhibit fungal growth or pathogenesis on or in plants or the ability to inhibit growth or lyse fungal cells *in vitro* in cell media as described herein. For example, in some embodiments, the anti-fungal activity is sufficient to inhibit fungal growth by at least 10%, sometimes 50%, and sometimes at least 90% compared to growth on media or plants without the polypeptide. Similarly, in some embodiments of the invention plants expressing polypeptides with anti-fungal activity have at least 10% fewer disease symptoms, sometimes 50% and sometimes at least 90% compared to equivalent plants not expressing the polypeptide. Symptoms will vary based on the fungal pathogen, but can include damping off of seedlings, lesion development or other symptoms known to those of skill in the art.

[0029] The phrase “nucleic acid sequence” refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5’ to the 3’ end. It includes chromosomal DNA, self-replicating plasmids and DNA or RNA that performs a primarily structural role.

[0030] The term “encoding” refers to a polynucleotide sequence encoding one or more amino acids. The term does not require a start or stop codon. An amino acid sequence can be encoded in any one of six different reading frames provided by a polynucleotide sequence.

[0031] The term “promoter” refers to regions or sequence located upstream and/or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription.

[0032] A “vector” refers to a polynucleotide, which when independent of the host chromosome, is capable of replication in a host organism. Examples of vectors include plasmids and typically have an origin of replication. Vectors can comprise, *e.g.*, transcription and translation terminators, transcription and translation initiation sequences, and promoters useful for regulation of the expression of the particular nucleic acid.

[0033] The term “plant” includes whole plants, shoots, vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (*e.g.* vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous

and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous plants.

[0034] A polynucleotide sequence is “heterologous to” an organism or a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (*e.g.* a genetically engineered coding sequence or an allele from a different ecotype or variety).

[0035] “Recombinant” refers to a human manipulated polynucleotide or a copy or complement of a human manipulated polynucleotide. For instance, a recombinant expression cassette comprising a promoter operably linked to a second polynucleotide may include a promoter that is heterologous to the second polynucleotide as the result of human manipulation (*e.g.*, by methods described in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)) of an isolated nucleic acid comprising the expression cassette. In another example, a recombinant expression cassette may comprise polynucleotides combined in such a way that the polynucleotides are extremely unlikely to be found in nature. For instance, human manipulated restriction sites or plasmid vector sequences may flank or separate the promoter from the second polynucleotide. One of skill will recognize that polynucleotides can be manipulated in many ways and are not limited to the examples above.

[0036] A polynucleotide “exogenous to” an individual plant is a polynucleotide which is introduced into the plant by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation, biolistic methods, electroporation, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T₁ (*e.g.* in *Arabidopsis* by vacuum infiltration) or R₀ (for plants regenerated from transformed cells *in vitro*) generation transgenic plant. Transgenic plants that arise from sexual cross or by selfing are descendants of such a plant.

[0037] A “chitinase nucleic acid” or “chitinase polynucleotide sequence” of the invention is a polynucleotide sequence or subsequence (*e.g.*, odd numbered sequences from SEQ ID NO: 3 to SEQ ID NO: 15 and SEQ ID NO: 21 to SEQ ID NO: 83) which, encodes a chitinase polypeptide (*e.g.*, even numbered sequences from SEQ ID NO: 4 to SEQ ID NO: 16 and SEQ

ID NO: 22 to SEQ ID NO: 84, respectively) with chitinase activity. “Chitinase” refers to a polypeptide capable of enzymatically hydrolyzing β -1,4 linked N-acetylglucosamine polymers (chitin). *See, e.g.,* Watanabe, *et al. Microbiology* 145(12):3353-63 (1999). Thus chitinase polypeptides inherently have “chitinase activity.”

[0038] “Chitinase nucleic acids” or “chitinase polynucleotide sequences” also include polynucleotides of at least about 10, or about 15, or about 20, or about 30, or about 50, or about 100 nucleotides in length that encode subsequences of the above-described chitinase polypeptides (*e.g.*, even numbered sequences from SEQ ID NO: 4 to SEQ ID NO: 16 and SEQ ID NO: 22 to SEQ ID NO: 84) that are not comprised in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 20. Chitinase polynucleotides are typically less than about 10,000 nucleotides, sometimes less than 5,000 nucleotides and sometimes less than 1,000 or 500 or 100 nucleotides in length.

[0039] Some chitinases of the invention exhibit improved chitinase activity as compared to the chitinase displayed in SEQ ID NO: 1 or SEQ ID NO: 2, in the assays described herein. A typical chitinase enzymatic assay consists of measuring the hydrolysis of carboxymethyl-chitin-remazol brilliant violet, as described herein. *See, e.g.,* Wirth and Wolf, *J. Microbiol. Methods* 12:197-205 (1990). Some chitinases of the invention exhibit an improvement of chitinase activity at least about 150% of the chitinase activity of SEQ ID NO: 1, more typically at least 200% of the activity of SEQ ID NO: 1, sometimes at least about 500% of the activity of SEQ ID NO: 1, sometimes at least 1,000% of activity of SEQ ID NO: 1, and sometimes at least 10,000% of the activity of SEQ ID NO: 1.

[0040] Other chitinase polypeptides of the invention have the same chitinase activity as or lower chitinase activity than SEQ ID NO: 1. Typically, these chitinases of the invention exhibit substantially the same activity as SEQ ID NO: 1. The polypeptides of the invention, however, can exhibit less than 70%, sometimes less than 50% and even less than 20% or less than 10% of the activity of SEQ ID NO: 1.

[0041] In the case of both expression of transgenes and inhibition of endogenous genes (*e.g.*, by antisense, or co-suppression) one of skill will recognize that the inserted polynucleotide sequence need not be identical, but may be only “substantially identical” to a sequence of the gene from which it was derived. As explained below, these substantially identical variants are specifically covered by the term “chitinase nucleic acid.”

[0042] In the case where the inserted polynucleotide sequence is transcribed and translated to produce a functional polypeptide, one of skill will recognize that because of codon degeneracy a

number of polynucleotide sequences will encode the same polypeptide. These variants are specifically covered by the terms “chitinase nucleic acid,” “chitinase polynucleotide” and their equivalents. In addition, the terms specifically include those full length sequences substantially identical and/or substantially similar (determined as described below) to a chitinase polynucleotide sequence and that encode proteins that retain the function of the chitinase polypeptide (*e.g.*, resulting from conservative substitutions of amino acids in the chitinase polypeptide).

[0043] As used herein, an “antibody” refers to a protein consisting of one or more polypeptides substantially or partially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kD) and one “heavy” chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively. Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)’₂, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)’₂ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (Fab)’₂ dimer into an Fab’ monomer. The Fab’ monomer is essentially an Fab with part of the hinge region (see, Fundamental Immunology, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab’ fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies. Antibodies include single chain antibodies, including single chain Fv

(sFv) antibodies in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide.

[0044] The phrase “specifically (or selectively) binds” to a polypeptide or “specifically (or selectively) immunoreactive with,” when referring to an antibody, refers to a binding reaction that is determinative of the presence of the polypeptide in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to a particular chitinase polypeptide of the invention can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with the polypeptide, and not with other proteins (*e.g.*, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20). This selection may be achieved by subtracting out antibodies that cross-react with other proteins. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g.*, Harlow & Lane, *Antibodies, A Laboratory Manual* (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background. Antibodies that react only with a particular chitinase polypeptide of the invention, *e.g.*, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82 or SEQ ID NO: 84 can also be made as described above, by subtracting out antibodies that bind to other chitinase proteins. For example, in a competitive binding assay between a polypeptide of the invention and a second polypeptide (*e.g.*, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, or SEQ ID NO: 20), it will require at least

ten times the amount of the polypeptide of the invention for the second polypeptide to inhibit 50% of the binding to the polypeptide of the invention.

[0045] Two nucleic acid sequences or polypeptides are said to be “identical” if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The terms “identical” or “percent identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (*e.g.*, charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated according to, *e.g.*, the algorithm of Meyers & Miller, *Computer Applic. Biol. Sci.* 4:11-17 (1988) *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA). The term “absolute percent identity” refers to a percentage of sequence identity determined by scoring identical amino acids as 1 and any substitution as zero, regardless of the similarity of mismatched amino acids. In a typical sequence alignment, *e.g.*, a BLAST alignment, the “absolute percent identity” of two sequences is presented as a percentage of amino acid “identities.” As used herein, where a sequence is defined as being “at least X% identical” to a reference sequence, *e.g.*, “a polypeptide at least 90% identical to SEQ ID NO: 4,” it is to be understood that “X% identical” refers to absolute percent identity, unless otherwise indicated. In cases where an optimal alignment of two sequences requires the insertion of a gap in one or both of the sequences, an amino acid residue in one sequence that aligns with a gap in

the other sequence is counted as a mismatch for purposes of determining percent identity. Gaps can be internal or external, *i.e.*, a truncation.

[0046] The term “substantial identity” of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 25% sequence identity. Alternatively, percent identity can be any integer from at least 25% to 100% (*e.g.*, at least 25%, 26%, 27%, 28%, ..., 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%). Some embodiments include at least: 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity compared to a reference sequence using the programs described herein; preferably BLAST using standard parameters, as described below. The present invention provides for polynucleotides that are at least substantially identical to SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81 or 83. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

[0047] These variant nucleotide sequences can also be evaluated by comparison of the percent sequence identity shared by the polypeptides they encode. For example, isolated nucleic acids which encode a polypeptide with a given percent sequence identity to the polypeptide of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82 or SEQ ID NO: 84 are disclosed. Identity can be calculated using, for example, the BLAST, CLUSTALW, or GAP algorithms under default conditions. The percentage of identity to a reference sequence is at least 50% and, rounded upwards to the nearest integer, can be expressed as an integer selected from the group of integers consisting of from 50 to 99. Thus, for example, the percentage of identity to a reference sequence can be at least 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

[0048] “Substantial identity” of amino acid sequences for these purposes normally means sequence identity of at least 40%. The percent identity of polypeptides can be any integer from at least 40% to 100% (e.g., at least 40%, 41%, 42%, 43%, ..., 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%). Some embodiments include at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity. The present invention provides for polypeptides that are at least substantially identical to SEQ ID NOS: 4, 6, 8, 10, 12, 14, 16, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82 or 84.

[0049] Polypeptides which are “substantially similar” share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Examples of conservative amino acid substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, aspartic acid-glutamic acid, and asparagine-glutamine.

[0050] The invention provides chitinase amino acid sequences that are “substantially similar” to any of SEQ ID NOS: 4, 6, 8, 10, 12, 14, 16, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82 or 84, as well as polynucleotides encoding these amino acid sequences. “Substantial similarity” of chitinase amino acid sequences can be determined by determining a similarity score for the two sequences. As used herein, the “similarity score” refers to the score generated for the two sequences using the BLOSUM62 amino acid substitution matrix, a gap existence penalty of 11, and a gap extension penalty of 1, when the two sequences are optimally aligned. Two sequences are “optimally aligned” when they are aligned so as to produce the maximum possible score for that pair of sequences, which might require the introduction of gaps in one or both of the sequences to achieve that maximum score. Two chitinase amino acid sequences are substantially similar if their similarity score exceeds a certain threshold value. The threshold

value can be any integer ranging from at least 1190 to the highest possible score for a particular reference chitinase sequence (*e.g.*, about 1500 for SEQ ID NO: 18). For example, the threshold similarity score can be 1190, 1200, 1210, 1220, 1230, 1240, 1250, 1260, 1270, 1280, 1290, 1300, 1310, 1320, 1330, 1340, 1350, 1360, 1370, 1380, 1390, 1400, 1410, 1420, 1430, 1440, 1450, 1460, 1470, 1480, 1490, 1500, or higher. If in a particular embodiment of the invention the threshold score is set at, for example, 1300, and the reference chitinase sequence is SEQ ID NO: 4, then any chitinase amino acid sequence that can be optimally aligned with SEQ ID NO: 4 to generate a similarity score of greater than 1300 is “substantially similar” to SEQ ID NO: 4.

[0051] Amino acid substitution matrices and their use in quantifying the similarity between two sequences are well-known in the art and described, *e.g.*, in Dayhoff *et al.* (1978), “A model of evolutionary change in proteins”, “Atlas of Protein Sequence and Structure,” Vol. 5, Suppl. 3 (ed. M.O. Dayhoff), pp. 345-352. Natl. Biomed. Res. Found., Washington, DC and in Henikoff *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919. A high similarity generally correlates with homology of the sequences. The BLOSUM62 matrix is often used as a default scoring substitution matrix in sequence alignment protocols such as Gapped BLAST 2.0. The gap existence penalty is imposed for opening gap in one of the aligned sequences, and the gap extension penalty is imposed for each amino acid position in the gap. Thus, a two amino acid residue gap will result in a penalty of 13, 11 for existence of the gap and 2 for extending the gap two amino acids. The alignment is defined by the amino acid positions of each sequence at which the alignment begins and ends, and optionally by the insertion of a gap or multiple gaps in one or both sequences, so as to arrive at the highest possible score. While optimal alignment and scoring can be accomplished manually, the process is facilitated by the use of a computer-implemented alignment algorithm, *e.g.*, gapped BLAST 2.0, described in Altschul *et al.*, (1997) Nucleic Acids Res. 25:3389-3402, and made available to the public at the National Center for Biotechnology Information website. To generate accurate similarity scores using NCBI BLAST, it is important to turn off any filtering, *e.g.*, low complexity filtering, and to disable the use of composition based statistics. One should also confirm that the correct substitution matrix and gap penalties are used. Optimal alignments, including multiple alignments, can be prepared using, *e.g.*, PSI-BLAST, available through the NCBI internet site and described by Altschul *et al.*, (1997) Nucleic Acids Res. 25:3389-3402. One of skill in the art will recognize that two polypeptides can also be “substantially identical” if the two polypeptides are immunologically similar. Thus, overall protein structure may be similar while the primary structure of the two

polypeptides display significant variation. Therefore a method to measure whether two polypeptides are substantially identical involves measuring the binding of monoclonal or polyclonal antibodies to each polypeptide. Two polypeptides are substantially identical if the antibodies specific for a first polypeptide bind to a second polypeptide with an affinity of at least one third of the affinity for the first polypeptide.

[0052] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0053] A “comparison window”, as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. When a “comparison window” is used to determine the percent identity between two sequences, a lower limit on the length of the comparison window can be imposed, *e.g.*, a minimum length of 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240 or 250 amino acids. Alternatively, percent identity can be defined such that the window of comparison over which the percent identity criterion is satisfied must include a sufficient amount of the reference sequence to possess some chitinase activity. Thus, if two sequences satisfy a minimum percent identity criterion (*e.g.*, at least 90% sequence identity) only over a window of comparison that is less than the entire length of the amino acid sequence used as a reference (*e.g.*, SEQ ID NO: 4), then the subsequence of the reference sequence corresponding to the window of comparison must itself have chitinase activity in order to conclude that the two sequences meet the percent identity criterion. For example, if two sequences only share X percent identity over a short window of comparison (*e.g.*, 20 contiguous amino acids), and that 20 contiguous amino acids is not sufficient unto itself to possess chitinase activity (which would normally be the case), then the two sequences do not satisfy the criterion of possessing at least X percent identity. Alternatively, if the window of comparison spans a longer subsequence of contiguous amino

acids that possesses chitinase activity even without the rest of the sequence, the criterion will be found to have been met.

[0054] Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

[0055] One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

[0056] Another example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when

aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al, supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0057] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0058] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally

identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0059] As to amino acid sequences, one of skill will recognize that individual substitutions, in a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

[0060] The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(see, e.g., Creighton, *Proteins* (1984)).

[0061] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below.

[0062] The phrase “selectively (or specifically) hybridizes to” refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

[0063] The phrase “stringent hybridization conditions” refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in*

Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). The T_m (thermal melting point) is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Generally, highly stringent conditions are selected to be about 5-10°C lower than the T_m for the specific sequence at a defined ionic strength pH. Low stringency conditions are generally selected to be about 15-30°C below the T_m . Hybridization conditions are typically those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0064] For the purposes of this disclosure, stringent conditions for such RNA-DNA hybridizations are those which include at least one wash in 0.2X SSC at 63°C for 20 minutes, or equivalent conditions. Genomic DNA or cDNA comprising genes of the invention can be identified using the polynucleotides explicitly disclosed herein (*e.g.*, odd numbered SEQ ID NO: s from 3-15 and 21-83), or fragments thereof of at least about 100 nucleotides, under stringent hybridization conditions. Stringent hybridization conditions, for purposes of this disclosure, include at least one wash (usually 2) in 0.2X SSC at a temperature of at least about 50°C, usually about 55°C, and sometimes 60°C or 65°C, for 20 minutes, or equivalent conditions. Exemplary high stringency conditions include hybridization in 50% formamide, 1M NaCl, 1% SDS at 37°C for at least 4 hours, more preferably up to 12 hours or longer, and a final wash in 0.1X SSC at 60 to 65°C for 30 minutes.

[0065] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice

background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

[0066] In the present invention, genomic DNA or cDNA comprising chitinase nucleic acids of the invention can also be identified in standard Southern blots under stringent conditions using the nucleic acid sequences disclosed herein. For the purposes of this disclosure, suitable stringent conditions for such hybridizations are those which include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and at least one wash in 0.2X SSC at a temperature of at least about 50°C, usually about 55°C to about 60°C, for 20 minutes, or equivalent conditions. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

[0067] A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, *e.g.*, an RNA gel or DNA gel blot hybridization analysis.

[0068] The present invention provides chitinase polypeptides, some of which possess improved enzymatic activity, *i.e.*, increased activity compared to the activity of the maize chitinase A (SEQ ID NO: 1). The invention also provides methods of improving plant resistance to fungal and nematode pathogens and other pests, such as insects. In particular, resistance to pests can be enhanced by introducing into plants a polynucleotide encoding a chitinase polypeptide of the invention.

[0069] The combination of alterations in the polypeptides of the invention result in a variety of levels of enzymatic activity. Thus, combinations of different alterations, which individually provide positive or negative effects, result in the ultimate variation in activity found in the polypeptides. For example, combinations of positive and negative (*i.e.*, inhibitory) alterations can lead to improved activity over wild type chitinase activity. In some cases, it is possible that alterations that produce a negative effect when added singly can have a positive, synergistic effect when combined with other alterations.

[0070] Polypeptides of the invention are related to the polypeptides exemplified in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16 and SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40,

SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82 or SEQ ID NO: 84. In some cases polypeptides of the invention exhibit improved chitinase activity compared to that of the maize chitinase A proteins, in accordance with the activity assays described herein. Table 1 illustrates the clone identities of the chitinases of the present invention.

Table 1

SEQ ID NO: (Nucleotide)	Clone ID	SEQ ID NO: (Polypeptide)	Clone ID
		SEQ ID NO: 1	chitinase A from maize
		SEQ ID NO: 2	Chitinase B from maize
SEQ ID NO: 3	R1B6	SEQ ID NO: 4	r1B6
SEQ ID NO: 5	R1B10	SEQ ID NO: 6	r1B10
SEQ ID NO: 7	R1δ	SEQ ID NO: 8	r1D4
SEQ ID NO: 9	R2Å	SEQ ID NO: 10	r2A2
SEQ ID NO: 11	R2C2	SEQ ID NO: 12	r2C2
SEQ ID NO: 13	R2E1	SEQ ID NO: 14	r2E1
SEQ ID NO: 15	R2H2	SEQ ID NO: 16	r2H2
		SEQ ID NO: 17	chitinase A, M84164
		SEQ ID NO: 18	Chitinase B, M84165
		SEQ ID NO: 19	chitinase A, Huynh <i>et al.</i>
		SEQ ID NO: 20	Chitinase B, Huynh <i>et al.</i>
SEQ ID NO: 21	4M1/11_C10	SEQ ID NO: 22	4M1/11_C10
SEQ ID NO: 23	4M1/1_H2	SEQ ID NO: 24	4M1/1_H2
SEQ ID NO: 25	4M1/26_C2	SEQ ID NO: 26	4M1/26_C2
SEQ ID NO: 27	R1AB2	SEQ ID NO: 28	r1AB2
SEQ ID NO: 29	R1AD4	SEQ ID NO: 30	r1AD4
SEQ ID NO: 31	R1AD6	SEQ ID NO: 32	r1AD6
SEQ ID NO: 33	R1AG9	SEQ ID NO: 34	r1AG9
SEQ ID NO: 35	R1AH8	SEQ ID NO: 36	r1AH8
SEQ ID NO: 37	R1AH9	SEQ ID NO: 38	r1AH9
SEQ ID NO: 39	R1BG5	SEQ ID NO: 40	r1BG5
SEQ ID NO: 41	R2C5	SEQ ID NO: 42	r2C5
SEQ ID NO: 43	4N1/95_H3	SEQ ID NO: 44	4N1/95_H3
SEQ ID NO: 45	4N1/80_F8	SEQ ID NO: 46	4N1/80_F8
SEQ ID NO: 47	4N1/75_D3	SEQ ID NO: 48	4N1/75_D3
SEQ ID NO: 49	4N1/2_H9	SEQ ID NO: 50	4N1/2_H9
SEQ ID NO: 51	4N1/23_G4	SEQ ID NO: 52	4N1/23_G4
SEQ ID NO: 53	4N1/68_E4	SEQ ID NO: 54	4N1/68_E4
SEQ ID NO: 55	4N1/88_F9	SEQ ID NO: 56	4N1/88_F9
SEQ ID NO: 57	4N1/14_B3	SEQ ID NO: 58	4N1/14_B3
SEQ ID NO: 59	4N1/33_F4	SEQ ID NO: 60	4N1/33_F4
SEQ ID NO: 61	4N1/11_B11	SEQ ID NO: 62	4N1/11_B11
SEQ ID NO: 63	4N1/30_D3	SEQ ID NO: 64	4N1/30_D3
SEQ ID NO: 65	4N1/35_G5	SEQ ID NO: 66	4N1/35_G5
SEQ ID NO: 67	4P1/2_B5	SEQ ID NO: 68	4P1/2_B5

SEQ ID NO: (Nucleotide)	Clone ID	SEQ ID NO: (Polypeptide)	Clone ID
SEQ ID NO: 69	4P1/7_H9	SEQ ID NO: 70	4P1/7_H9
SEQ ID NO: 71	4Q1/3_H10	SEQ ID NO: 72	4Q1/3_H10
SEQ ID NO: 73	4Q1/5_B11	SEQ ID NO: 74	4Q1/5_B11
SEQ ID NO: 75	4Q2/10_B8	SEQ ID NO: 76	4Q2/10_B8
SEQ ID NO: 77	4Q2/13_F8	SEQ ID NO: 78	4Q2/13_F8
SEQ ID NO: 79	4R2/1_G10	SEQ ID NO: 80	4R2/1_G10
SEQ ID NO: 81	4R2/5_H11	SEQ ID NO: 82	4R2/5_H11
SEQ ID NO: 83	4R2/9_B2	SEQ ID NO: 84	4R2/9_B2

[0071] The amino acid sequences of the polypeptides of the invention comprise at least one amino acid difference from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 20. The amino acid sequences of the chitinases exemplified in the application (*e.g.*, even numbered sequences from SEQ ID NO: 4 to 16 and SEQ ID NO: 22-84) each have improved activity over maize Chitinases A (SEQ ID NO: 1). Therefore, chitinases comprising some or all of the differences between the exemplified sequences and chitinase A are likely to increase enzymatic activity of a chitinase polypeptide.

[0072] The present invention provides isolated nucleic acids comprising a polynucleotide encoding a chitinase polypeptide, wherein the chitinase polypeptide is selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84. The chitinase polypeptides of the present invention exhibit a chitinase activity of at least 20% of the chitinase activity of chitinase A (SEQ ID NO: 1) to at least 200% of the chitinase activity of chitinase A (SEQ ID NO: 1).

[0073] The invention also provides for isolated nucleic acids comprising a polynucleotide encoding a chitinase polypeptide, wherein the chitinase polypeptide is substantially identical and/or substantially similar to a polypeptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID

NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84. For example, the invention provides for isolated nucleic acids comprising a polynucleotide encoding a chitinase polypeptide, wherein the chitinase polypeptide is selected from the group consisting of a polypeptide at least 90% identical to SEQ ID NO: 4, at least 89% identical to SEQ ID NO: 6, at least 91% identical to SEQ ID NO: 8, at least 88% identical to SEQ ID NO: 10, at least 91% identical to SEQ ID NO: 12, at least 89% identical to SEQ ID NO: 14, at least 87% identical to SEQ ID NO: 16, at least 96% identical to SEQ ID NO: 22, at least 90% identical to SEQ ID NO: 24, at least 89% identical to SEQ ID NO: 26, at least 92% identical to SEQ ID NO: 28, at least 92% identical to SEQ ID NO: 30, at least 95% identical to SEQ ID NO: 32, at least 92% identical to SEQ ID NO: 34, at least 95% identical to SEQ ID NO: 36, at least 93% identical to SEQ ID NO: 38, at least 90% identical to SEQ ID NO: 40, at least 90% identical to SEQ ID NO: 42, at least 96% identical to SEQ ID NO: 44, at least 94% identical to SEQ ID NO: 46, at least 95% identical to SEQ ID NO: 48, at least 96% identical to SEQ ID NO: 50, at least 99% identical to SEQ ID NO: 52, at least 95% identical to SEQ ID NO: 54, at least 95% identical to SEQ ID NO: 56, at least 93% identical to SEQ ID NO: 58, at least 93% identical to SEQ ID NO: 60, at least 93% identical to SEQ ID NO: 62, at least 93% identical to SEQ ID NO: 64, at least 93% identical to SEQ ID NO: 66, a polypeptide at least 91% identical to SEQ ID NO: 68, a polypeptide at least 92% identical to SEQ ID NO: 70, a polypeptide at least 90% identical to SEQ ID NO: 72, a polypeptide at least 89% identical to SEQ ID NO: 74, a polypeptide at least 89% identical to SEQ ID NO: 76, a polypeptide at least 93% identical to SEQ ID NO: 78, a polypeptide at least 91% identical to SEQ ID NO: 80, a polypeptide at least 92% identical to SEQ ID NO: 82, and a polypeptide at least 91% identical to SEQ ID NO: 84. The isolated nucleic acids of the present invention encode chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises one or more amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. Further it is understood that the isolated nucleic acids of the present

invention encode chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more or ten amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises lysine at position 86, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 124, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 83, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises valine at position 85, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 97, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 99, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises arginine at position 238, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 230, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 79, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses

isolated nucleic acids encoding chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises aspartic acid at position 219, wherein the position corresponds to that in SEQ ID NO: 70. With respect to an amino acid sequence that is optimally aligned with a reference sequence, an amino acid residue “corresponds to” the position in the reference sequence with which the residue is paired in an alignment. The “position” is denoted by a number that sequentially identifies each amino acid in the reference sequence based on its position relative to the N-terminus. These chitinase polypeptides have a chitinase activity of at least 20% of the chitinase activity of chitinase A (SEQ ID NO: 1) to at least 200% of the chitinase activity of chitinase A (SEQ ID NO: 1).

[0074] The present invention provides isolated nucleic acids comprising a chitinase polynucleotide encoding a polypeptide having chitinase activity, wherein the polynucleotide is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, and SEQ ID NO: 83.

[0075] In some embodiments, the nucleic acid further comprises a promoter operably linked to the polynucleotide. The promoter may be a tissue-specific promoter, a constitutive promoter or an inducible promoter. The present invention also provides vectors comprising a nucleic acid of the invention operably linked to a promoter, which may be tissue-specific, constitutive or inducible.

[0076] The present invention also provides isolated nucleic acids comprising a chitinase polynucleotide encoding a polypeptide with chitinase activity, wherein the polynucleotide specifically hybridizes following at least one wash in 0.1X SSC at 60° C for 30 minutes to a probe polynucleotide selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65,

SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, and SEQ ID NO: 83, with the proviso that the chitinase polynucleotide does not encode SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 20. The polypeptides exhibit a chitinase activity of at least 20% of the chitinase activity of chitinase A (SEQ ID NO: 1) to at least 200% of the chitinase activity of chitinase A (SEQ ID NO: 1). In some embodiments, the isolated nucleic acid does not encode SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, or SEQ ID NO: 16.

[0077] The present invention also provides isolated nucleic acids of at least 20 nucleotides in length, wherein the nucleic acid encodes an amino acid subsequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84, with the proviso that the nucleic acid does not encode an amino acid subsequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 20. In some embodiments, the isolated nucleic acid does not encode an amino acid subsequence of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 or SEQ ID NO: 16.

[0078] The present invention provides isolated chitinase polypeptides selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84.

[0079] The present invention also provides an isolated chitinase polypeptide selected from the group consisting of a polypeptide at least 90% identical to SEQ ID NO: 4, a polypeptide at

least 89% identical to SEQ ID NO: 6, a polypeptide at least 91% identical to SEQ ID NO: 8, a polypeptide at least 88% identical to SEQ ID NO: 10, a polypeptide at least 91% identical to SEQ ID NO: 12, a polypeptide at least 89% identical to SEQ ID NO: 14, a polypeptide at least 87% identical to SEQ ID NO: 16, a polypeptide at least 96% identical to SEQ ID NO: 22, a polypeptide at least 90% identical to SEQ ID NO: 24, a polypeptide at least 89% identical to SEQ ID NO: 26, a polypeptide at least 92% identical to SEQ ID NO: 28, a polypeptide at least 92% identical to SEQ ID NO: 30, a polypeptide at least 95% identical to SEQ ID NO: 32, a polypeptide at least 92% identical to SEQ ID NO: 34, a polypeptide at least 95% identical to SEQ ID NO: 36, a polypeptide at least 93% identical to SEQ ID NO: 38, a polypeptide at least 90% identical to SEQ ID NO: 40, a polypeptide at least 90% identical to SEQ ID NO: 42, a polypeptide at least 96% identical to SEQ ID NO: 44, a polypeptide at least 94% identical to SEQ ID NO: 46, a polypeptide at least 95% identical to SEQ ID NO: 48, a polypeptide at least 96% identical to SEQ ID NO: 50, a polypeptide at least 99% identical to SEQ ID NO: 52, a polypeptide at least 95% identical to SEQ ID NO: 54, a polypeptide at least 95% identical to SEQ ID NO: 56, a polypeptide at least 93% identical to SEQ ID NO: 58, a polypeptide at least 93% identical to SEQ ID NO: 60, a polypeptide at least 93% identical to SEQ ID NO: 62, a polypeptide at least 93% identical to SEQ ID NO: 64, a polypeptide at least 93% identical to SEQ ID NO: 66, a polypeptide at least 91% identical to SEQ ID NO: 68, a polypeptide at least 92% identical to SEQ ID NO: 70, a polypeptide at least 90% identical to SEQ ID NO: 72, a polypeptide at least 89% identical to SEQ ID NO: 74, a polypeptide at least 89% identical to SEQ ID NO: 76, a polypeptide at least 93% identical to SEQ ID NO: 78, a polypeptide at least 91% identical to SEQ ID NO: 80, a polypeptide at least 92% identical to SEQ ID NO: 82, and a polypeptide at least 91% identical to SEQ ID NO: 84. Further, the chitinase polypeptides of the present invention have an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises one or more amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. Further it is understood that the present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more or ten amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position

86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises lysine at position 86, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 124, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 83, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises valine at position 85, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 97, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises arginine at position 238, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 230, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 79, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises aspartic acid at position 219, wherein the position corresponds to that in SEQ ID NO: 70. With respect to an amino acid sequence that is optimally aligned with a reference sequence, an amino acid residue “corresponds to” the position in the reference sequence with which the residue is paired in an alignment. The “position” is denoted by a number that sequentially identifies each amino acid in the reference sequence based on its position relative to the N-terminus. These polypeptides exhibit a chitinase

activity of at least 20% of the chitinase activity of chitinase A (SEQ ID NO: 1) to at least 200% of the chitinase activity of chitinase A (SEQ ID NO: 1).

[0080] The present invention also provides antibodies capable of specifically binding an isolated polypeptide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84.

[0081] The present invention also provides plants comprising a recombinant expression cassette comprising a promoter operably linked to a polynucleotide encoding a chitinase polypeptide having chitinase activity, wherein the polypeptide is selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84. The chitinase polypeptides exhibit a chitinase activity of at least 20% of the chitinase activity of chitinase A (SEQ ID NO: 1) to at least 200% of the chitinase activity of chitinase A (SEQ ID NO: 1). In some embodiments, the promoter is a tissue-specific promoter, while in other embodiments the promoter is a constitutive promoter, while yet in other embodiments the promoter is an inducible promoter. In some embodiments, the plant is maize.

[0082] The present invention also provides plants comprising a recombinant expression cassette comprising a promoter operably linked to a polynucleotide encoding a chitinase polypeptide with chitinase activity, wherein the polypeptide is selected from the group consisting of a polypeptide at least 90% identical to SEQ ID NO: 4, a polypeptide at least 89% identical to SEQ ID NO: 6, a polypeptide at least 91% identical to SEQ ID NO: 8, a polypeptide at least 88% identical to SEQ ID NO: 10, a polypeptide at least 91% identical to SEQ ID NO: 12, a polypeptide at least 89% identical to SEQ ID NO: 14, a polypeptide at least 87%

identical to SEQ ID NO: 16, a polypeptide at least 96% identical to SEQ ID NO: 22, a polypeptide at least 90% identical to SEQ ID NO: 24, a polypeptide at least 89% identical to SEQ ID NO: 26, a polypeptide at least 92% identical to SEQ ID NO: 28, a polypeptide at least 92% identical to SEQ ID NO: 30, a polypeptide at least 95% identical to SEQ ID NO: 32, a polypeptide at least 92% identical to SEQ ID NO: 34, a polypeptide at least 95% identical to SEQ ID NO: 36, a polypeptide at least 93% identical to SEQ ID NO: 38, a polypeptide at least 90% identical to SEQ ID NO: 40, a polypeptide at least 90% identical to SEQ ID NO: 42, a polypeptide at least 96% identical to SEQ ID NO: 44, a polypeptide at least 94% identical to SEQ ID NO: 46, a polypeptide at least 95% identical to SEQ ID NO: 48, a polypeptide at least 96% identical to SEQ ID NO: 50, a polypeptide at least 99% identical to SEQ ID NO: 52, a polypeptide at least 95% identical to SEQ ID NO: 54, a polypeptide at least 95% identical to SEQ ID NO: 56, a polypeptide at least 93% identical to SEQ ID NO: 58, a polypeptide at least 93% identical to SEQ ID NO: 60, a polypeptide at least 93% identical to SEQ ID NO: 62, a polypeptide at least 93% identical to SEQ ID NO: 64, a polypeptide at least 93% identical to SEQ ID NO: 66, a polypeptide at least 91% identical to SEQ ID NO: 68, a polypeptide at least 92% identical to SEQ ID NO: 70, a polypeptide at least 90% identical to SEQ ID NO: 72, a polypeptide at least 89% identical to SEQ ID NO: 74, a polypeptide at least 89% identical to SEQ ID NO: 76, a polypeptide at least 93% identical to SEQ ID NO: 78, a polypeptide at least 91% identical to SEQ ID NO: 80, a polypeptide at least 92% identical to SEQ ID NO: 82, and a polypeptide at least 91% identical to SEQ ID NO: 84. Further, the plants of the present invention contain chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises one or more amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. Further it is understood that the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more or ten amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. The present

invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises lysine at position 86, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 124, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 83, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises valine at position 85, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 97, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 99, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises arginine at position 238, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 230, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 79, wherein the position corresponds to that in SEQ ID NO: 70. The present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises aspartic acid at position 219, wherein the position corresponds to that in SEQ ID NO: 70. With respect to an amino acid sequence that is optimally aligned with a reference sequence, an amino acid residue "corresponds to" the position in the reference sequence with which the residue is paired in an alignment. The "position" is denoted by a number that sequentially identifies each amino acid in the reference sequence based on its position relative to the N-terminus. These chitinase polypeptides exhibit a chitinase activity of at least 20% of the chitinase activity of chitinase A (SEQ ID NO: 1) to at least 200% of the chitinase activity of chitinase A (SEQ ID

NO: 1). In some embodiments, the promoter is a tissue-specific promoter, while in other embodiments the promoter is a constitutive promoter, while yet in other embodiments the promoter is an inducible promoter. In some embodiments, the plant is maize.

[0083] The present invention also provides methods of enhancing plant resistance to a fungus. The method comprises a) introducing into a plant a recombinant expression cassette comprising a promoter operably linked to a polynucleotide encoding a chitinase polypeptide having chitinase activity, wherein the polypeptide is selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84; and b) selecting a plant with enhanced resistance to a fungus. In some embodiments, the promoter is a tissue-specific promoter, while in other embodiments the promoter is a constitutive promoter, and in yet other embodiments the promoter is an inducible promoter. In some embodiments, the plant is maize. Further, in some embodiments, the fungus is from the genus *Fusarium*.

[0084] The present invention also provides methods of enhancing plant resistance to a fungus comprising a) introducing into a plant a recombinant expression cassette comprising a promoter operably linked to a polynucleotide encoding a chitinase polypeptide having chitinase activity, wherein the polypeptide is selected from the group consisting of a polypeptide at least 90% identical to SEQ ID NO: 4, a polypeptide at least 89% identical to SEQ ID NO: 6, a polypeptide at least 91% identical to SEQ ID NO: 8, a polypeptide at least 88% identical to SEQ ID NO: 10, a polypeptide at least 91% identical to SEQ ID NO: 12, a polypeptide at least 89% identical to SEQ ID NO: 14, a polypeptide at least 87% identical to SEQ ID NO: 16, a polypeptide at least 96% identical to SEQ ID NO: 22, a polypeptide at least 90% identical to SEQ ID NO: 24, a polypeptide at least 89% identical to SEQ ID NO: 26, a polypeptide at least 92% identical to SEQ ID NO: 28, a polypeptide at least 92% identical to SEQ ID NO: 30, a polypeptide at least 95% identical to SEQ ID NO: 32, a polypeptide at least 92% identical to SEQ ID NO: 34, a polypeptide at least 95% identical to SEQ ID NO: 36, a polypeptide at least 93% identical to SEQ ID NO: 38, a polypeptide at least 90% identical to SEQ ID NO: 40, a

polypeptide at least 90% identical to SEQ ID NO: 42, a polypeptide at least 96% identical to SEQ ID NO: 44, a polypeptide at least 94% identical to SEQ ID NO: 46, a polypeptide at least 95% identical to SEQ ID NO: 48, a polypeptide at least 96% identical to SEQ ID NO: 50, a polypeptide at least 99% identical to SEQ ID NO: 52, a polypeptide at least 95% identical to SEQ ID NO: 54, a polypeptide at least 95% identical to SEQ ID NO: 56, a polypeptide at least 93% identical to SEQ ID NO: 58, a polypeptide at least 93% identical to SEQ ID NO: 60, a polypeptide at least 93% identical to SEQ ID NO: 62, a polypeptide at least 93% identical to SEQ ID NO: 64, a polypeptide at least 93% identical to SEQ ID NO: 66, a polypeptide at least 91% identical to SEQ ID NO: 68, a polypeptide at least 92% identical to SEQ ID NO: 70, a polypeptide at least 90% identical to SEQ ID NO: 72, a polypeptide at least 89% identical to SEQ ID NO: 74, a polypeptide at least 89% identical to SEQ ID NO: 76, a polypeptide at least 93% identical to SEQ ID NO: 78, a polypeptide at least 91% identical to SEQ ID NO: 80, a polypeptide at least 92% identical to SEQ ID NO: 82, and a polypeptide at least 91% identical to SEQ ID NO: 84; and b) selecting a plant with enhanced resistance to a fungus. Further, the method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises one or more amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. Further it is understood that the method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more or ten amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises lysine at position 86, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 124, wherein the position corresponds to that in SEQ ID

NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 83, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises valine at position 85, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 97, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 99, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises arginine at position 238, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 230, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 79, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises aspartic acid at position 219, wherein the position corresponds to that in SEQ ID NO: 70. With respect to an amino acid sequence that is optimally aligned with a reference sequence, an amino acid residue “corresponds to” the position in the reference sequence with which the residue is paired in an alignment. The “position” is denoted by a number that sequentially identifies each amino acid in the reference sequence based on its position relative to the N-terminus. In some embodiments, the promoter is a tissue-specific promoter, while in other embodiments the promoter is a constitutive promoter, and in yet other embodiments the promoter is an inducible promoter. In some embodiments, the plant is maize. In some embodiments, the fungus is from the genus *Fusarium*.

[0085] The present invention also provides methods of enhancing plant resistance to a nematode. The method comprises a) introducing into a plant a recombinant expression cassette

comprising a promoter operably linked to a polynucleotide encoding a chitinase polypeptide having chitinase activity, wherein the polypeptide is selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, and SEQ ID NO: 84; and b) selecting a plant with enhanced resistance to a nematode. In some embodiments, the promoter is a tissue-specific promoter, while in other embodiments the promoter is a constitutive promoter, and in yet other embodiments the promoter is an inducible promoter. In some embodiments, the plant is soybean. Further, in some embodiments, the nematode is from the genus *Heterodera*.

[0086] The present invention also provides methods of enhancing plant resistance to a nematode comprising a) introducing into a plant a recombinant expression cassette comprising a promoter operably linked to a polynucleotide encoding a chitinase polypeptide having chitinase activity, wherein the polypeptide is selected from the group consisting of a polypeptide at least 90% identical to SEQ ID NO: 4, a polypeptide at least 89% identical to SEQ ID NO: 6, a polypeptide at least 91% identical to SEQ ID NO: 8, a polypeptide at least 88% identical to SEQ ID NO: 10, a polypeptide at least 91% identical to SEQ ID NO: 12, a polypeptide at least 89% identical to SEQ ID NO: 14, a polypeptide at least 87% identical to SEQ ID NO: 16, a polypeptide at least 96% identical to SEQ ID NO: 22, a polypeptide at least 90% identical to SEQ ID NO: 24, a polypeptide at least 89% identical to SEQ ID NO: 26, a polypeptide at least 92% identical to SEQ ID NO: 28, a polypeptide at least 92% identical to SEQ ID NO: 30, a polypeptide at least 95% identical to SEQ ID NO: 32, a polypeptide at least 92% identical to SEQ ID NO: 34, a polypeptide at least 95% identical to SEQ ID NO: 36, a polypeptide at least 93% identical to SEQ ID NO: 38, a polypeptide at least 90% identical to SEQ ID NO: 40, a polypeptide at least 90% identical to SEQ ID NO: 42, a polypeptide at least 96% identical to SEQ ID NO: 44, a polypeptide at least 94% identical to SEQ ID NO: 46, a polypeptide at least 95% identical to SEQ ID NO: 48, a polypeptide at least 96% identical to SEQ ID NO: 50, a polypeptide at least 99% identical to SEQ ID NO: 52, a polypeptide at least 95% identical to SEQ ID NO: 54, a polypeptide at least 95% identical to SEQ ID NO: 56, a polypeptide at least

93% identical to SEQ ID NO: 58, a polypeptide at least 93% identical to SEQ ID NO: 60, a polypeptide at least 93% identical to SEQ ID NO: 62, a polypeptide at least 93% identical to SEQ ID NO: 64, a polypeptide at least 93% identical to SEQ ID NO: 66, a polypeptide at least 91% identical to SEQ ID NO: 68, a polypeptide at least 92% identical to SEQ ID NO: 70, a polypeptide at least 90% identical to SEQ ID NO: 72, a polypeptide at least 89% identical to SEQ ID NO: 74, a polypeptide at least 89% identical to SEQ ID NO: 76, a polypeptide at least 93% identical to SEQ ID NO: 78, a polypeptide at least 91% identical to SEQ ID NO: 80, a polypeptide at least 92% identical to SEQ ID NO: 82, and a polypeptide at least 91% identical to SEQ ID NO: 84; and b) selecting a plant with enhanced resistance to a nematode. Further, the method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises one or more amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. Further it is understood that the method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more or ten amino acid residues selected from the group consisting of serine at position 79, serine at position 83, valine at position 85, lysine at position 86, glutamine at position 97, glutamine at position 99, isoleucine at position 124, aspartic acid at position 219, isoleucine at position 230, and arginine at position 238, wherein the positions correspond to those in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises lysine at position 86, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 124, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 83, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises

valine at position 85, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 97, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises glutamine at position 99, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises arginine at position 238, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises isoleucine at position 230, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises serine at position 79, wherein the position corresponds to that in SEQ ID NO: 70. The method of the present invention encompasses plants containing chitinase polypeptides having an amino acid sequence that, when optimally aligned with SEQ ID NO: 70 comprises aspartic acid at position 219, wherein the position corresponds to that in SEQ ID NO: 70. With respect to an amino acid sequence that is optimally aligned with a reference sequence, an amino acid residue “corresponds to” the position in the reference sequence with which the residue is paired in an alignment. The “position” is denoted by a number that sequentially identifies each amino acid in the reference sequence based on its position relative to the N-terminus. In some embodiments, the promoter is a tissue-specific promoter, while in other embodiments the promoter is a constitutive promoter, and in yet other embodiments the promoter is an inducible promoter. In some embodiments, the plant is soybean. In some embodiments, the fungus is from the genus *Heterodera*.

[0087] Polypeptides of the invention can optionally encompass a signal sequence to target the polypeptides of the invention to a particular organelle or compartment of the cell.

[0088] Either naturally occurring or recombinant chitinase polypeptides can be purified for use in functional assays. Naturally occurring chitinase polypeptides can be purified, *e.g.*, from plant tissue and any other source of a chitinase. Recombinant chitinase polypeptides can be purified from any suitable expression system.

[0089] The chitinase polypeptides may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (see, *e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)(Ausubel *et al.*)).

[0090] A number of procedures can be employed when recombinant chitinase polypeptides are being purified. For example, proteins having established molecular adhesion properties can be reversibly fused to the chitinase polypeptides. With the appropriate ligand, the chitinase polypeptides can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein can then be removed by enzymatic activity. Finally the chitinase polypeptides could be purified using immunoaffinity columns.

[0091] Immunoassays in a competitive binding format can be used to identify polypeptide sequences with cross reactivity to an antibody raised to a particular polypeptide or epitope of the invention. For example, a protein at least partially encoded by an odd numbered sequence between SEQ ID NO: 3 and SEQ ID NO: 15 or SEQ ID NO: 21 and SEQ ID NO: 83, or an immunogenic region thereof, can be immobilized to a solid support. Other competitor proteins such as SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 18 or maize chitinases A and B described in Huynh, *et al.*, *J. Biol. Chem.* 267:6635-6640 (1992) (SEQ ID NO: 19 or SEQ ID NO: 20) or modifications or fragments thereof, can be added to the assay so as to compete for binding of the antisera to the immobilized antigen. The ability of the added proteins to compete for binding of the antisera to the immobilized protein is compared to the ability of the particular polypeptide of the invention (*e.g.*, even-numbered sequences from SEQ ID NO: 4 to SEQ ID NO: 16 and SEQ ID NO: 22 to SEQ ID NO: 84) to compete with itself. In some cases, at least one protein as displayed in even-number sequences between SEQ ID NO: 4 and SEQ ID NO: 16 is used as a competitor protein. The percent cross-reactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% cross-reactivity with each of the added competitor proteins listed above are selected and pooled. The cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with the added considered competitor proteins, *e.g.*, distantly related homologs, or other homologs of the polypeptide of the invention (*e.g.*, SEQ ID NO: 1, SEQ ID NO: 2 (*see, e.g.*, PCT WO 00/56908), SEQ ID NO: 17 (Genbank Accession No. M84164) and SEQ ID NO: 18 (Genbank

Accession No. M84165), SEQ ID NO: 19 and SEQ ID NO: 20 (*see, e.g., Huynh, et al., J. Biol. Chem.* 267:6635-6640 (1992)).

[0092] The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described below to compare a second protein, thought to be perhaps an allele or polymorphic variant of the particular chitinase, to the immunogen protein. In order to make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein required to inhibit 50% of binding is less than 10 times the amount of the chitinase polypeptide of the invention that is required to inhibit 50% of binding, then the second protein is said to specifically bind to the polyclonal antibodies generated to the respective chitinase immunogen.

[0093] In competitive assays, the amount of the chitinase present in the sample is measured indirectly by measuring the amount of known, added (exogenous) chitinase displaced (competed away) from an anti-chitinase antibody by the unknown chitinase present in a sample. In one competitive assay, a known amount of the chitinase is added to a sample and the sample is then contacted with an antibody that specifically binds to the chitinase. The amount of exogenous chitinase bound to the antibody is inversely proportional to the concentration of the chitinase present in the sample. In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of chitinase bound to the antibody may be determined either by measuring the amount of chitinase present in a chitinase/antibody complex, or alternatively by measuring the amount of remaining uncomplexed protein. The amount of chitinase may be detected by providing a labeled chitinase molecule.

[0094] A hapten inhibition assay is another preferred competitive assay. In this assay the known chitinase is immobilized on a solid substrate. A known amount of anti-chitinase antibody is added to the sample, and the sample is then contacted with the immobilized chitinase. The amount of anti-chitinase antibody bound to the known immobilized chitinase is inversely proportional to the amount of chitinase present in the sample. Again, the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

[0095] Nucleic acids of the invention generally comprise all or part of a polynucleotide encoding a chitinase polypeptide of the invention.

[0096] Nucleic acids of the invention also encompass nucleic acid probes. Probes are useful, for instance, to detect differences between maize chitinase genes A and B (SEQ ID NOS: 1 and 2) and polynucleotides encoding alterations in the maize chitinase A and B sequences that give rise to improved or altered enzymatic activity. Such alterations can result from, *e.g.*, insertions, deletions or point mutations. In some embodiments, the alterations are linear combinations of SEQ ID NO: 1 and SEQ ID NO: 2. Probes can be of any length useful to detect a desired polynucleotide.

[0097] In one aspect of the invention, probes are designed to bind to the polynucleotides of the invention at sequences comprised of fusions of subsequences of SEQ ID NO: 1 and SEQ ID NO: 2. For example, nucleotides 498 to 776 of SEQ ID NO: 3 comprises nucleotides 613 to 767 of SEQ ID NO: 1 and nucleotides 468 to 594 of SEQ ID NO: 2. For example, those of skill in the art will recognize that probes can be designed to selectively hybridize to a polynucleotide encoding the polypeptides or polypeptide subsequences of the invention but not hybridize to the native maize chitinase A or B polynucleotide sequences (SEQ ID NOS: 1 and 2) or SEQ ID NOS: 17 or 18.

[0098] Chitinases or enzymatically functional equivalents thereof can be constructed synthetically by using the polymerase chain reaction (PCR), either independently of the cloning vector used (Dillon, *et al. BioTechniques* 9:298-300 (1990); Sandhu, *et al. BioTechniques* 12:12-16 (1992)) or by direct cloning into a vector (Ivanov, *et al. Gene* 95:295-299 (1990); Foguet, *et al. BioTechniques* 13:674-675 (1992)). Alternatively, complete genes can be constructed from synthetic PCR fragments or duplex oligonucleotides through in-frame cloning (Pierce, J. C. *Methods Mol. Biol.* 67:151-65 (1997)).

[0099] Chitinase polynucleotides of the invention can be readily modified using methods that are well known in the art to improve or alter chitinase activity. A variety of diversity generating protocols are available and described in the art. The procedures can be used separately, and/or in combination to produce one or more variants of a nucleic acid or set of nucleic acids, as well as variants of encoded proteins. Individually and collectively, these procedures provide robust, widely applicable ways of generating diversified nucleic acids and sets of nucleic acids (including, nucleic acid libraries) which are useful, for the engineering or rapid evolution of nucleic acids, proteins, pathways, cells and/or organisms with new and/or improved characteristics.

[0100] While distinctions and classifications are made in the course of the ensuing discussion for clarity, it will be appreciated that the techniques are often not mutually exclusive. Indeed,

the various methods can be used singly or in combination, in parallel or in series, to access diverse sequence variants.

[0101] The result of any of the diversity generating procedures described herein can be the generation of one or more nucleic acids, which can be selected or screened for nucleic acids that encode proteins with or which confer desirable properties. Following diversification by one or more of the methods herein, or otherwise available to one of skill, any nucleic acids that are produced can be selected for a desired activity or property, *e.g.* chitinase activity. This can include identifying any activity that can be detected, for example, in an automated or automatable format, by any of the assays in the art, for example, by assaying the hydrolysis of carboxymethyl-chitin-remazol brilliant violet, as described herein. A variety of related (or even unrelated) properties can be evaluated, in serial or in parallel, at the discretion of the practitioner.

[0102] Descriptions of a variety of diversity generating procedures for generating modified chitinase nucleic acid sequences of the invention are found in the following publications and the references cited therein: Stemmer, *et al.* (1999) "Molecular breeding of viruses for targeting and other clinical properties" *Tumor Targeting* 4:1-4; Ness *et al.* (1999) "DNA Shuffling of subgenomic sequences of subtilisin" *Nature Biotechnology* 17:893-896; Chang *et al.* (1999) "Evolution of a cytokine using DNA family shuffling" *Nature Biotechnology* 17:793-797; Minshull and Stemmer (1999) "Protein evolution by molecular breeding" *Current Opinion in Chemical Biology* 3:284-290; Christians *et al.* (1999) "Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling" *Nature Biotechnology* 17:259-264; Crameri *et al.* (1998) "DNA shuffling of a family of genes from diverse species accelerates directed evolution" *Nature* 391:288-291; Crameri *et al.* (1997) "Molecular evolution of an arsenate detoxification pathway by DNA shuffling," *Nature Biotechnology* 15:436-438; Zhang *et al.* (1997) "Directed evolution of an effective fucosidase from a galactosidase by DNA shuffling and screening" *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Patten *et al.* (1997) "Applications of DNA Shuffling to Pharmaceuticals and Vaccines" *Current Opinion in Biotechnology* 8:724-733; Crameri *et al.* (1996) "Construction and evolution of antibody-phage libraries by DNA shuffling" *Nature Medicine* 2:100-103; Crameri *et al.* (1996) "Improved green fluorescent protein by molecular evolution using DNA shuffling" *Nature Biotechnology* 14:315-319; Gates *et al.* (1996) "Affinity selective isolation of ligands from peptide libraries through display on a lac repressor 'headpiece dimer'" *Journal of Molecular Biology* 255:373-386; Stemmer (1996) "Sexual PCR and Assembly PCR" In: *The Encyclopedia of Molecular Biology*. VCH Publishers, New York. pp. 447-457; Crameri and Stemmer (1995) "Combinatorial

multiple cassette mutagenesis creates all the permutations of mutant and wildtype cassettes” *BioTechniques* 18:194-195; Stemmer *et al.*, (1995) “Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxy-ribonucleotides” *Gene*, 164:49-53; Stemmer (1995) “The Evolution of Molecular Computation” *Science* 270: 1510; Stemmer (1995) “Searching Sequence Space” *Bio/Technology* 13:549-553; Stemmer (1994) “Rapid evolution of a protein *in vitro* by DNA shuffling” *Nature* 370:389-391; and Stemmer (1994) “DNA shuffling by random fragmentation and reassembly: *In vitro* recombination for molecular evolution.” *Proc. Natl. Acad. Sci. USA* 91:10747-10751.

[0103] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Ling *et al.* (1997) “Approaches to DNA mutagenesis: an overview” *Anal. Biochem.* 254(2): 157-178; Dale *et al.* (1996) “Oligonucleotide-directed random mutagenesis using the phosphorothioate method” *Methods Mol. Biol.* 57:369-374; Smith (1985) “*In vitro* mutagenesis” *Ann. Rev. Genet.* 19:423-462; Botstein & Shortle (1985) “Strategies and applications of *in vitro* mutagenesis” *Science* 229:1193-1201; Carter (1986) “Site-directed mutagenesis” *Biochem. J.* 237:1-7; and Kunkel (1987) “The efficiency of oligonucleotide directed mutagenesis” in *Nucleic Acids & Molecular Biology* (Eckstein, F. and Lilley, D.M.J. eds., Springer Verlag, Berlin)); mutagenesis using uracil containing templates (Kunkel (1985) “Rapid and efficient site-specific mutagenesis without phenotypic selection” *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) “Rapid and efficient site-specific mutagenesis without phenotypic selection” *Methods in Enzymol.* 154, 367-382; and Bass *et al.* (1988) “Mutant Trp repressors with new DNA-binding specificities” *Science* 242:240-245); oligonucleotide-directed mutagenesis (*Methods in Enzymol.* 100: 468-500 (1983); *Methods in Enzymol.* 154: 329-350 (1987); Zoller & Smith (1982) “Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any DNA fragment” *Nucleic Acids Res.* 10:6487-6500; Zoller & Smith (1983) “Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors” *Methods in Enzymol.* 100:468-500; and Zoller & Smith (1987) “Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template” *Methods in Enzymol.* 154:329-350); phosphorothioate-modified DNA mutagenesis (Taylor *et al.* (1985) “The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA” *Nucl. Acids Res.* 13: 8749-8764; Taylor *et al.* (1985) “The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA” *Nucl. Acids Res.* 13: 8765-8787 (1985); Nakamaye & Eckstein (1986) “Inhibition of restriction

endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis" *Nucl. Acids Res.* 14: 9679-9698; Sayers *et al.* (1988) "Y-T Exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis" *Nucl. Acids Res.* 16:791-802; and Sayers *et al.* (1988) "Strand specific cleavage of phosphorothioate-containing DNA by reaction with restriction endonucleases in the presence of ethidium bromide" *Nucl. Acids Res.* 16: 803-814); mutagenesis using gapped duplex DNA (Kramer *et al.* (1984) "The gapped duplex DNA approach to oligonucleotide-directed mutation construction" *Nucl. Acids Res.* 12: 9441-9456; Kramer & Fritz (1987) *Methods in Enzymol.* "Oligonucleotide-directed construction of mutations via gapped duplex DNA" 154:350-367; Kramer *et al.* (1988) "Improved enzymatic *in vitro* reactions in the gapped duplex DNA approach to oligonucleotide-directed construction of mutations" *Nucl. Acids Res.* 16: 7207; and Fritz *et al.* (1988) "Oligonucleotide-directed construction of mutations: a gapped duplex DNA procedure without enzymatic reactions *in vitro*" *Nucl. Acids Res.* 16: 6987-6999).

[0104] Additional suitable methods include point mismatch repair (Kramer *et al.* (1984) "Point Mismatch Repair" *Cell* 38:879-887), mutagenesis using repair-deficient host strains (Carter *et al.* (1985) "Improved oligonucleotide site-directed mutagenesis using M13 vectors" *Nucl. Acids Res.* 13: 4431-4443; and Carter (1987) "Improved oligonucleotide-directed mutagenesis using M13 vectors" *Methods in Enzymol.* 154: 382-403), deletion mutagenesis (Eghtedarzadeh & Henikoff (1986) "Use of oligonucleotides to generate large deletions" *Nucl. Acids Res.* 14: 5115), restriction-selection and restriction-selection and restriction-purification (Wells *et al.* (1986) "Importance of hydrogen-bond formation in stabilizing the transition state of subtilisin" *Phil. Trans. R. Soc. Lond. A* 317: 415-423), mutagenesis by total gene synthesis (Nambiar *et al.* (1984) "Total synthesis and cloning of a gene coding for the ribonuclease S protein" *Science* 223: 1299-1301; Sakamar and Khorana (1988) "Total synthesis and expression of a gene for the a-subunit of bovine rod outer segment guanine nucleotide-binding protein (transducin)" *Nucl. Acids Res.* 14: 6361-6372; Wells *et al.* (1985) "Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites" *Gene* 34:315-323; and Grundström *et al.* (1985) "Oligonucleotide-directed mutagenesis by microscale 'shot-gun' gene synthesis" *Nucl. Acids Res.* 13: 3305-3316), double-strand break repair (Mandecki (1986); Arnold (1993) "Protein engineering for unusual environments" *Current Opinion in Biotechnology* 4:450-455. "Oligonucleotide-directed double-strand break repair in plasmids of *Escherichia coli*: a method for site-specific mutagenesis" *Proc. Natl. Acad. Sci. USA*, 83:7177-7181). Additional details on many of the above methods can be found in *Methods in*

Enzymology Volume 154, which also describes useful controls for trouble-shooting problems with various mutagenesis methods.

[0105] Additional details regarding various diversity generating methods can be found in the following U.S. patents, PCT publications, and EPO publications: U.S. Pat. No. 5,605,793 to Stemmer (February 25, 1997), "Methods for *In vitro* Recombination;" U.S. Pat. No. 5,811,238 to Stemmer *et al.* (September 22, 1998) "Methods for Generating Polynucleotides having Desired Characteristics by Iterative Selection and Recombination;" U.S. Patent No. 5,830,721 to Stemmer *et al.* (November 3, 1998), "DNA Mutagenesis by Random Fragmentation and Reassembly;" U.S. Pat. No. 5,834,252 to Stemmer, *et al.* (November 10, 1998) "End-Complementary Polymerase Reaction;" U.S. Pat. No. 5,837,458 to Minshull, *et al.* (November 17, 1998), "Methods and Compositions for Cellular and Metabolic Engineering;" WO 95/22625, Stemmer and Cramer, "Mutagenesis by Random Fragmentation and Reassembly;" WO 96/33207 by Stemmer and Lipschutz "End Complementary Polymerase Chain Reaction;" WO 97/20078 by Stemmer and Cramer "Methods for Generating Polynucleotides having Desired Characteristics by Iterative Selection and Recombination;" WO 97/35966 by Minshull and Stemmer, "Methods and Compositions for Cellular and Metabolic Engineering;" WO 99/41402 by Punnonen *et al.* "Targeting of Genetic Vaccine Vectors;" WO 99/41383 by Punnonen *et al.* "Antigen Library Immunization;" WO 99/41369 by Punnonen *et al.* "Genetic Vaccine Vector Engineering;" WO 99/41368 by Punnonen *et al.* "Optimization of Immunomodulatory Properties of Genetic Vaccines;" EP 752008 by Stemmer and Cramer, "DNA Mutagenesis by Random Fragmentation and Reassembly;" EP 0932670 by Stemmer "Evolving Cellular DNA Uptake by Recursive Sequence Recombination;" WO 99/23107 by Stemmer *et al.*, "Modification of Virus Tropism and Host Range by Viral Genome Shuffling;" WO 99/21979 by Apt *et al.*, "Human Papillomavirus Vectors;" WO 98/31837 by del Cardayre *et al.* "Evolution of Whole Cells and Organisms by Recursive Sequence Recombination;" WO 98/27230 by Patten and Stemmer, "Methods and Compositions for Polypeptide Engineering;" WO 98/13487 by Stemmer *et al.*, "Methods for Optimization of Gene Therapy by Recursive Sequence Shuffling and Selection;" WO 00/00632, "Methods for Generating Highly Diverse Libraries;" WO 00/09679, "Methods for Obtaining *in vitro* Recombined Polynucleotide Sequence Banks and Resulting Sequences;" WO 98/42832 by Arnold *et al.*, "Recombination of Polynucleotide Sequences Using Random or Defined Primers;" WO 99/29902 by Arnold *et al.*, "Method for Creating Polynucleotide and Polypeptide Sequences;" WO 98/41653 by Vind, "An *in vitro* Method for Construction of a DNA Library;" WO 98/41622 by Borchert *et al.*, "Method for

Constructing a Library Using DNA Shuffling,” and WO 98/42727 by Pati and Zarling, “Sequence Alterations using Homologous Recombination.”

[0106] Certain U.S. applications provide additional details regarding various diversity generating methods, including “Shuffling of Codon Altered Genes” by Patten *et al.* filed September 28, 1999, (USSN 09/407,800); “Evolution of Whole Cells and Organisms by Recursive Sequence Recombination”, by del Cardayre *et al.* filed July 15, 1998 (USSN 09/166,188), and July 15, 1999 (USSN 09/354,922); “Oligonucleotide Mediated Nucleic Acid Recombination” by Crameri *et al.*, filed September 28, 1999 (USSN 09/408,392), and “Oligonucleotide Mediated Nucleic Acid Recombination” by Crameri *et al.*, filed January 18, 2000 (PCT/US00/01203); “Use of Codon-Based Oligonucleotide Synthesis for Synthetic Shuffling” by Welch *et al.*, filed September 28, 1999 (USSN 09/408,393); “Methods for Making Character Strings, Polynucleotides & Polypeptides Having Desired Characteristics” by Selifonov *et al.*, filed January 18, 2000, (PCT/US00/01202) and, *e.g.*, “Methods for Making Character Strings, Polynucleotides & Polypeptides Having Desired Characteristics” by Selifonov *et al.*, filed July 18, 2000 (USSN 09/618,579); “Methods of Populating Data Structures for Use in Evolutionary Simulations” by Selifonov and Stemmer (USSN PCT/US00/01138), filed January 18, 2000; and “Single-Stranded Nucleic Acid Template-Mediated Recombination and Nucleic Acid Fragment Isolation” by Affholter, USSN 60/186,482, filed March 2, 2000.

[0107] In brief, several different general classes of sequence modification methods, such as mutation, recombination, *etc.* are applicable to the present invention and set forth in the references above. Accordingly, the chitinase nucleic acids of the invention can be generated from wild type sequences. Moreover, the chitinase nucleic acid sequences of the invention can be modified to create modified sequences with the same or different activity.

The following illustrate some exemplary formats for diversity generation in the context of the present invention, including, certain recombination based diversity generation formats.

Nucleic acids can be recombined *in vitro* by any of a variety of techniques discussed in the references above, including, DNase digestion of nucleic acids to be recombined followed by ligation and/or PCR reassembly of the nucleic acids. For example, sexual PCR mutagenesis can be used in which random (or pseudo random, or even non-random) fragmentation of the DNA molecule is followed by recombination, based on sequence similarity, between DNA molecules with different but related DNA sequences, *in vitro*, followed by fixation of the crossover by extension in a polymerase chain reaction. This process and many process variants is described

in several of the references above, such as, in Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751. Thus, nucleic acids encoding chitinase with modified activity can be generated.

[0108] Similarly, nucleic acids can be recursively recombined *In vivo* by allowing recombination to occur between nucleic acids in cells. Many such *In vivo* recombination formats are set forth in the references noted above. Such formats optionally provide direct recombination between nucleic acids of interest, or provide recombination between vectors, viruses, plasmids, *etc.*, comprising the nucleic acids of interest, as well as other formats. Details regarding such procedures are found in the references noted above.

[0109] Whole genome recombination methods can also be used in which whole genomes of cells or other organisms are recombined, optionally including spiking of the genomic recombination mixtures with desired library components (for example, genes corresponding to the pathways of the present invention). These methods have many applications, including those in which the identity of a target gene is not known. Details on such methods are found in WO 98/31837 by del Cardayre *et al.* "Evolution of Whole Cells and Organisms by Recursive Sequence Recombination" and in PCT/US99/15972 by del Cardayre *et al.*, also entitled "Evolution of Whole Cells and Organisms by Recursive Sequence Recombination."

[0110] Synthetic recombination methods can also be used, in which oligonucleotides corresponding to targets of interest are synthesized and reassembled in PCR or ligation reactions which include oligonucleotides which correspond to more than one parental nucleic acid, thereby generating new recombined nucleic acids. Oligonucleotides can be made by standard nucleotide addition methods or can be made by tri-nucleotide synthetic approaches. Details regarding such approaches are found in the references noted above, including, "Oligonucleotide Mediated Nucleic Acid Recombination" by Crameri *et al.*, filed September 28, 1999 (USSN 09/408,392), and "Oligonucleotide Mediated Nucleic Acid Recombination" by Crameri *et al.*, filed January 18, 2000 (PCT/US00/01203); "Use of Codon-Based Oligonucleotide Synthesis for Synthetic Shuffling" by Welch *et al.*, filed September 28, 1999 (USSN 09/408,393); "Methods for Making Character Strings, Polynucleotides & Polypeptides Having Desired Characteristics" by Selifonov *et al.*, filed January 18, 2000, (PCT/US00/01202); "Methods of Populating Data Structures for Use in Evolutionary Simulations" by Selifonov and Stemmer (PCT/US00/01138), filed January 18, 2000; and, *e.g.*, "Methods for Making Character Strings, Polynucleotides & Polypeptides Having Desired Characteristics" by Selifonov *et al.*, filed July 18, 2000 (USSN 09/618,579).

[0111] In silico methods of recombination can be effected in which genetic algorithms are used in a computer to recombine sequence strings which correspond to homologous (or even non-homologous) nucleic acids. The resulting recombined sequence strings are optionally converted into nucleic acids by synthesis of nucleic acids which correspond to the recombined sequences, in concert with oligonucleotide synthesis/gene reassembly techniques. This approach can generate random, partially random or designed variants. Many details regarding in silico recombination, including the use of genetic algorithms, genetic operators and the like in computer systems, combined with generation of corresponding nucleic acids (and/or proteins), as well as combinations of designed nucleic acids and/or proteins (for example, based on cross-over site selection) as well as designed, pseudo-random or random recombination methods are described in "Methods for Making Character Strings, Polynucleotides & Polypeptides Having Desired Characteristics" by Selifonov *et al.*, filed January 18, 2000, (PCT/US00/01202) "Methods of Populating Data Structures for Use in Evolutionary Simulations" by Selifonov and Stemmer (PCT/US00/01138), filed January 18, 2000; and, *e.g.*, "Methods for Making Character Strings, Polynucleotides & Polypeptides Having Desired Characteristics" by Selifonov *et al.*, filed July 18, 2000 (USSN 09/618,579). Extensive details regarding in silico recombination methods are found in these applications. This methodology is generally applicable to the present invention in providing for recombination of the chitinase nucleic acids in silico and/or the generation of corresponding nucleic acids or proteins.

[0112] Many methods of accessing natural diversity, such as, by hybridization of diverse nucleic acids or nucleic acid fragments to single-stranded templates, followed by polymerization and/or ligation to regenerate full-length sequences, optionally followed by degradation of the templates and recovery of the resulting modified nucleic acids can be similarly used. In one method employing a single-stranded template, the fragment population derived from the genomic library(ies) is annealed with partial, or, often approximately full length ssDNA or RNA corresponding to the opposite strand. Assembly of complex chimeric genes from this population is then mediated by nuclease-base removal of non-hybridizing fragment ends, polymerization to fill gaps between such fragments and subsequent single stranded ligation. The parental polynucleotide strand can be removed by digestion (if RNA or uracil-containing), magnetic separation under denaturing conditions (if labeled in a manner conducive to such separation) and other available separation/purification methods. Alternatively, the parental strand is optionally co-purified with the chimeric strands and removed during subsequent screening and processing steps. Additional details regarding this approach are found, in "Single-Stranded Nucleic Acid

Template-Mediated Recombination and Nucleic Acid Fragment Isolation” by Affholter, USSN 60/186,482, filed March 2, 2000.

[0113] In another approach, single-stranded molecules are converted to double-stranded DNA (dsDNA) and the dsDNA molecules are bound to a solid support by ligand-mediated binding. After separation of unbound DNA, the selected DNA molecules are released from the support and introduced into a suitable host cell to generate a library of enriched sequences which hybridize to the probe. A library produced in this manner provides a desirable substrate for further diversification using any of the procedures described herein.

[0114] Any of the preceding general recombination formats can be practiced in a reiterative fashion (*e.g.*, one or more cycles of mutation/recombination or other diversity generation methods, optionally followed by one or more selection methods) to generate a more diverse set of recombinant nucleic acids.

[0115] Mutagenesis employing polynucleotide chain termination methods have also been proposed (*see e.g.*, U.S. Patent No. 5,965,408, “Method of DNA reassembly by interrupting synthesis” to Short, and the references above), and can be applied to the present invention. In this approach, double stranded DNAs corresponding to one or more genes sharing regions of sequence similarity are combined and denatured, in the presence or absence of primers specific for the gene. The single stranded polynucleotides are then annealed and incubated in the presence of a polymerase and a chain terminating reagent (*e.g.*, ultraviolet, gamma or X-ray irradiation; ethidium bromide or other intercalators; DNA binding proteins, such as single strand binding proteins, transcription activating factors, or histones; polycyclic aromatic hydrocarbons; trivalent chromium or a trivalent chromium salt; or abbreviated polymerization mediated by rapid thermocycling; and the like), resulting in the production of partial duplex molecules. The partial duplex molecules, containing partially extended chains, are then denatured and reannealed in subsequent rounds of replication or partial replication resulting in polynucleotides which share varying degrees of sequence similarity and which are diversified with respect to the starting population of DNA molecules. Optionally, the products, or partial pools of the products, can be amplified at one or more stages in the process. Polynucleotides produced by a chain termination method, such as described above, are suitable substrates for any other described recombination format.

[0116] Diversity also can be generated in nucleic acids or populations of nucleic acids using a recombinational procedure termed “incremental truncation for the creation of hybrid enzymes” (“ITCHY”) described in Ostermeier *et al.* (1999) “A combinatorial approach to hybrid enzymes

independent of DNA homology” *Nature Biotech* 17:1205. This approach can be used to generate an initial library of variants which can optionally serve as a substrate for one or more *in vitro* or *In vivo* recombination methods. See, also, Ostermeier *et al.* (1999) “Combinatorial Protein Engineering by Incremental Truncation,” *Proc. Natl. Acad. Sci. USA*, 96: 3562-67; and Ostermeier *et al.* (1999), “Incremental Truncation as a Strategy in the Engineering of Novel Biocatalysts,” *Biological and Medicinal Chemistry*, 7: 2139-44.

[0117] Mutational methods which result in the alteration of individual nucleotides or groups of contiguous or non-contiguous nucleotides can be favorably employed to introduce nucleotide diversity. Thus, modified chitinase nucleic acids of the invention can be generated, including for optimized codon usage for an organism of interest, as well as nucleic acids encoding chitinase polypeptides with improved and/or modified activity. Many mutagenesis methods are found in the above-cited references; and additional details regarding mutagenesis methods can be found in the references discussed below, which can also be applied to the present invention.

[0118] For example, error-prone PCR can be used to generate nucleic acid variants. Using this technique, PCR is performed under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Examples of such techniques are found in the references above and, in Leung *et al.* (1989) *Technique* 1:11-15 and Caldwell *et al.* (1992) *PCR Methods Applic.* 2:28-33. Similarly, assembly PCR can be used, in a process which involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions can occur in parallel in the same reaction mixture, with the products of one reaction priming the products of another reaction.

[0119] Oligonucleotide directed mutagenesis can be used to introduce site-specific mutations in a nucleic acid sequence of interest. Examples of such techniques are found in the references above and in Reidhaar-Olson *et al.* (1988) *Science*, 241:53-57. Similarly, cassette mutagenesis can be used in a process that replaces a small region of a double stranded DNA molecule with a synthetic oligonucleotide cassette that differs from the native sequence. The oligonucleotide can contain, completely and/or partially randomized native sequence(s).

[0120] Recursive ensemble mutagenesis is a process in which an algorithm for protein mutagenesis is used to produce diverse populations of phenotypically related mutants, members of which differ in amino acid sequence. This method uses a feedback mechanism to monitor successive rounds of combinatorial cassette mutagenesis. Examples of this approach are found in Arkin & Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815.

[0121] Exponential ensemble mutagenesis can be used for generating combinatorial libraries with a high percentage of unique and functional mutants. Small groups of residues in a sequence of interest are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Examples of such procedures are found in Delegrave & Youvan (1993) *Biotechnology Research* 11:1548-1552.

[0122] *In vivo* mutagenesis can be used to generate random mutations in any cloned DNA of interest by propagating the DNA in a strain of *E. coli* that carries mutations in one or more of the DNA repair pathways. These “mutator” strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA. Such procedures are described in the references noted above.

[0123] Other procedures for introducing diversity into a genome, *e.g.*, a bacterial, fungal, animal or plant genome, can be used in conjunction with the above described and/or referenced methods. For example, in addition to the methods above, techniques have been proposed which produce nucleic acid multimers suitable for transformation into a variety of species (*see*, for example, Schellenberger U.S. Patent No. 5,756,316 and the references above). Transformation of a suitable host with such multimers, consisting of genes that are divergent with respect to one another, (*e.g.*, derived from natural diversity or through application of site directed mutagenesis, error prone PCR, passage through mutagenic bacterial strains, and the like), provides a source of nucleic acid diversity for DNA diversification, by an *In vivo* recombination process as indicated above.

[0124] Alternatively, a multiplicity of monomeric polynucleotides sharing regions of partial sequence similarity can be transformed into a host species and recombined *In vivo* by the host cell. Subsequent rounds of cell division can be used to generate libraries, members of which, include a single, homogenous population, or pool of monomeric polynucleotides. Alternatively, the monomeric nucleic acid can be recovered by standard techniques, such as PCR and/or cloning, and recombined in any of the recombination formats, including recursive recombination formats, described above.

[0125] Methods for generating multispecies expression libraries have been described (in addition to the reference noted above, *see, e.g.*, Peterson *et al.* (1998) U.S. Pat. No. 5,783,431 “Methods for Generating and Screening Novel Metabolic Pathways,” and Thompson, *et al.* (1998) U.S. Patent No. 5,824,485 Methods for Generating and Screening Novel Metabolic Pathways) and their use to identify protein activities of interest has been proposed (in addition to

the references noted above, *see*, Short (1999) U.S. Pat. No. 5,958,672 “Protein Activity Screening of Clones Having DNA from Uncultivated Microorganisms”). Multispecies expression libraries include, in general, libraries comprising cDNA or genomic sequences from a plurality of species or strains, operably linked to appropriate regulatory sequences, in an expression cassette. The cDNA and/or genomic sequences are optionally randomly ligated to further enhance diversity. The vector can be a shuttle vector suitable for transformation and expression in more than one species of host organism, for example, bacterial species, eukaryotic cells. In some cases, the library is biased by preselecting sequences which encode a protein of interest, or which hybridize to a nucleic acid of interest. Any such libraries can be provided as substrates for any of the methods herein described.

[0126] The above described procedures have been largely directed to increasing nucleic acid and/or encoded protein diversity. However, in many cases, not all of the diversity is useful, for example, functional, and contributes merely to increasing the background of variants that must be screened or selected to identify the few favorable variants. In some applications, it is desirable to preselect or prescreen libraries (*e.g.*, an amplified library, a genomic library, a cDNA library, a normalized library, *etc.*) or other substrate nucleic acids prior to diversification, for example, by recombination-based mutagenesis procedures, or to otherwise bias the substrates towards nucleic acids that encode functional products. For example, in the case of antibody engineering, it is possible to bias the diversity generating process toward antibodies with functional antigen binding sites by taking advantage of *In vivo* recombination events prior to manipulation by any of the described methods. For example, recombined complementarity determining regions (CDRs) derived from B cell cDNA libraries can be amplified and assembled into framework regions (*see*, Jirholt *et al.* (1998) “Exploiting sequence space: shuffling *In vivo* formed complementarity determining regions into a master framework” *Gene* 215: 471) prior to diversifying according to any of the methods described herein.

[0127] Libraries can be biased towards nucleic acids which encode proteins with desirable enzyme activities. For example, after identifying a clone from a library that exhibits a specified activity, the clone can be mutagenized using any known method for introducing DNA alterations. A library comprising the mutagenized homologues is then screened for a desired activity, which can be the same as or different from the initially specified activity. An example of such a procedure is proposed in Short (1999) U.S. Patent No. 5,939,250 for “Production of Enzymes Having Desired Activities by Mutagenesis.” Desired activities can be identified by any method known in the art. For example, WO 99/10539 proposes that gene libraries can be

screened by combining extracts from the gene library with components obtained from metabolically rich cells and identifying combinations which exhibit the desired activity. It has also been proposed (see, WO 98/58085) that clones with desired activities can be identified by inserting bioactive substrates into samples of the library, and detecting bioactive fluorescence corresponding to the product of a desired activity using a fluorescent analyzer, such as, a flow cytometry device, a charge couple device (CCD), a fluorometer, or a spectrophotometer.

[0128] Libraries can also be biased towards nucleic acids which have specified characteristics, for example, hybridization to a selected nucleic acid probe. In particular, application WO 99/10539 proposes that polynucleotides encoding a desired activity (for example, an enzymatic activity, such as, a lipase, an esterase, a protease, a glycosidase, a glycosyl transferase, a phosphatase, a kinase, an oxygenase, a peroxidase, a hydrolase, a hydratase, a nitrilase, a transaminase, an amidase or an acylase) can be identified from among genomic DNA sequences in the following manner. Single stranded DNA molecules from a population of genomic DNA are hybridized to a ligand-conjugated probe. The genomic DNA can be derived from either a cultivated or uncultivated microorganism, or from an environmental sample. Alternatively, the genomic DNA can be derived from a multicellular organism or a tissue derived therefrom. Second strand synthesis can be conducted directly from the hybridization probe used in the capture, with or without prior release from the capture medium or by a wide variety of other strategies known in the art. Alternatively, the isolated single-stranded genomic DNA population can be fragmented without further cloning and used directly in a recombination-based approach that employs a single-stranded template, as described above.

[0129] "Non-Stochastic" methods of generating nucleic acids and polypeptides are alleged in Short "Non-Stochastic Generation of Genetic Vaccines and Enzymes" WO 00/46344. These methods, including proposed non-stochastic polynucleotide reassembly and site-saturation mutagenesis methods can be applied to the present invention as well.

[0130] It will readily be appreciated that any of the above described techniques, which are suitable for enriching a library prior to diversification can also be used to screen the products, or libraries of products, produced by the diversity generating methods.

[0131] Kits for mutagenesis, library construction and other diversity generation methods are also commercially available. For example, kits are available from, for example, Stratagene (QuickChange™ site-directed mutagenesis kit; and Chameleon™ double-stranded, site-directed mutagenesis kit), Bio/Can Scientific, Bio-Rad (using the Kunkel method described above), Boehringer Mannheim Corp., Clontech Laboratories, DNA Technologies, Epicentre

Technologies (5 prime 3 prime kit); Genpak Inc, Lemargo Inc, Life Technologies (Gibco BRL), New England Biolabs, Pharmacia Biotech, Promega Corp., Quantum Biotechnologies, Amersham International plc (using the Eckstein method above), and Anglian Biotechnology Ltd (using the Carter/Winter method above).

[0132] The above references provide many mutational formats, including recombination, recursive recombination, recursive mutation and combinations or recombination with other forms of mutagenesis, as well as many modifications of these formats. Regardless of the diversity generation format that is used, the nucleic acids of the invention can be recombined (with each other, or with related (or even unrelated) sequences) to produce a diverse set of recombinant nucleic acids, including, for example, sets of homologous nucleic acids, as well as corresponding polypeptides.

[0133] The polynucleotide sequence encoding a particular chitinase can be altered to coincide with the codon usage of a particular host. For example, the codon usage of a monocot plant can be used to derive a polynucleotide that encodes a chitinase polypeptide of the invention and comprises preferred monocot codons. The frequency of preferred codon usage exhibited by a host cell can be calculated by averaging the frequency of preferred codon usage in a large number of genes expressed by the host cell. This analysis is preferably limited to genes that are highly expressed by the host cell. U.S. Patent No. 5,824,864, for example, provides the frequency of codon usage by highly expressed genes exhibited by dicotyledonous plants and monocotyledonous plants.

[0134] When synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell. The percent deviation of the frequency of preferred codon usage for a synthetic gene from that employed by a host cell is calculated first by determining the percent deviation of the frequency of usage of a single codon from that of the host cell followed by obtaining the average deviation over all codons.

[0135] A method was developed by which yeast libraries could be screened for the functional expression of cloned chitinases. A plate-clearing assay (*see*, Wirth and Wolf, *J. Microbiol. Methods* 12:197-205 (1990)) was developed for determining the hydrolytic activity of chitinases. Briefly, a yeast strain (*Pichia pastoris* KM-71 (Invitrogen)) was modified by two rounds of EMS mutagenesis, in order to reduce the clearing zones generated by KM-71 colonies grown on CM-chitin-RBV-containing agar plates. After transformation of the mutagenized *Pichia pastoris* strain with libraries of chitinases or chitinase variants or fragments, functional

enzymes can be identified by the presence of a clearing zone surrounding the colonies successfully expressing functional chitinases. The identified clones are then selected for subsequent characterization. The selected clones are grown in liquid and the expressed recombinant protein activity is determined in kinetic assays. Such kinetic assays can, for example, involve CM-chitin-RBV, or colloidal chitin as substrates (Wirth and Wolf, *J. Microbiol. Methods* 12:197-205 (1990); Reissig *et al.*, *J. Biol. Chem.* 217:959-966 (1955); Legrand *et al.*, *Proc. Natl. Acad. Sci. USA* 84:6750-6754 (1987)). Specific activity measurements are then taken in substrate-saturating conditions.

[0136] General methods to perform assays to test the effect of chitinases for anti-fungal activity are known in the art. These assays include both *In vivo* and *in vitro* methods of testing a polypeptide for anti-fungal activity.

[0137] *In vivo* methods for testing for anti-fungal activity include expressing a candidate chitinase polypeptide in a plant and then growing the plant in the presence of a fungal pathogen. Improved plant health of the transformant relative to an untransformed control indicates that the polypeptide has anti-fungal activity. Alternatively, fungal populations either in plant tissue or on the exterior of the plant or in the surrounding soil can be measured relative to the tissues or soil associated with untransformed control plants.

[0138] *In vitro* methods of assaying for antifungal activity include zone clearing assays, in which fungal spores or hyphae are allowed to grow on a solid growth medium. Chitinases are added to the growth medium, for example, by deposition onto filter paper disks, or by direct addition to wells that were previously formed in the medium. Alternatively, chitinases can be secreted by an expression host maintained on the same medium as the fungal pathogen. Antifungal activity is evidenced by the formation of a clearing zone around the chitinase or the chitinase-producing host. Antifungal activity can also be monitored in liquid format, preferably in microtiter plates. In this case, a purified chitinase, or a crude protein mix containing chitinase, is assayed in a liquid growth medium, in which fungal spores were allowed to germinate. The efficiency of the chitinase at preventing the growth of the fungal pathogen is evidenced by the turbidity of the liquid growth medium (absorbance measurement).

[0139] Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These

techniques and various other techniques are generally performed according to Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998) ("Ausubel *et al.*").

[0140] The isolation of chitinase nucleic acids may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired organ, such as leaves, and a cDNA library which contains a chitinase gene transcript is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which chitinase genes or homologues are expressed.

[0141] The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned chitinase gene disclosed here. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. Alternatively, antibodies raised against a chitinase polypeptide can be used to screen an mRNA expression library.

[0142] Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of chitinase genes directly from genomic DNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. For a general overview of PCR, see *PCR Protocols: A Guide to Methods and Applications*. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), *Academic Press*, San Diego (1990). Appropriate primers and probes for identifying chitinase sequences from plant tissues are generated from comparisons of the sequences provided here (odd numbered sequences between SEQ ID NO: 3 and SEQ ID NO: 15 and between SEQ ID NO: 21 and SEQ ID NO: 83).

[0143] Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. See, e.g., Carruthers *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams *et al.*, *J. Am. Chem. Soc.* 105:661 (1983). Double stranded DNA

fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

[0144] One useful method to produce the nucleic acids of the invention is to isolate and modify the wild type maize chitinase polynucleotide sequences displayed in SEQ ID NO: 1 and SEQ ID NO: 2. Other sequences that can be modified include SEQ ID NO: 17 and SEQ ID NO: 18. Several methods for sequence-specific mutagenesis of a nucleic acid are known and are described above. In addition, Ausubel *et al.*, *supra*, describes oligonucleotide-directed mutagenesis as well as directed mutagenesis of nucleic acids using PCR. Such methods are useful to insert specific codon changes into the wild type maize chitinase A or B polynucleotide sequences, thereby constructing the nucleic acids of the invention. Basic cloning and PCR methods are also useful in combining subsequences of SEQ ID NO: 1 and SEQ ID NO: 2 to produce the polynucleotides of the invention.

[0145] The nucleic acid sequences of the present invention can be expressed in a host cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

[0146] Typical vectors contain transcription and translation terminators, transcription and translation initiation sequences, and promoters useful for regulation of the expression of the particular nucleic acid. The vectors optionally comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the cassette in eukaryotes, or prokaryotes, or both, (such as, shuttle vectors) and selection markers for both prokaryotic and eukaryotic systems. Vectors are suitable for replication and integration in prokaryotes, eukaryotes, or both. See, Gilman & Smith, *Gene* 8:81 (1979); Roberts, *et al.*, *Nature*, 328:731 (1987); Schneider, B., *et al.*, *Protein Expr. Purif.* 6435:10 (1995); Berger, Sambrook, Ausubel (all *supra*). A catalogue of Bacteria and Bacteriophages useful for cloning is provided by the ATCC, The ATCC Catalogue of Bacteria and Bacteriophage (1992) Gherna *et al.* (eds). Additional basic procedures for sequencing, cloning and other aspects of molecular biology and underlying theoretical considerations are also found in Watson *et al.* (1992) RECOMBINANT DNA Second Edition Scientific American Books, NY.

[0147] To use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, for example, Weising *et al. Ann. Rev. Genet.* 22:421-477 (1988). In some embodiments a DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, is combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant. Native or heterologous promoters can be operatively linked to transcriptional sequences.

[0148] Specifically, the chitinase sequences of the invention are provided in expression cassettes or DNA constructs for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a chitinase sequence of the invention. By "operably linked" a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence is intended. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

[0149] Such an expression cassette is provided with a plurality of restriction sites for insertion of the chitinase sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

[0150] The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a chitinase DNA sequence of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" it is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

[0151] While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of

chitinase in the host cell (*i.e.*, plant or plant cell). Thus, the phenotype of the host cell (*i.e.*, plant or plant cell) is altered.

[0152] The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See, Guérineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfaçon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

[0153] Where appropriate, as described above, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

[0154] Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. As discussed above, the G-C content of the sequence may be adjusted to levels that are average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

[0155] The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include, but are not limited to: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *PNAS USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986) *Virology* 154:9-20); MDMV leader (Maize Dwarf Mosaic Virus); human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, Ed., Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also, Della-

Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

[0156] In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.*, transitions and transversions, may be involved.

[0157] Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glyphosate, glufosinate, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struct. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschmidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334:721-724 and U.S. Patent Application Serial No. 10/072,307. Such disclosures are herein incorporated by reference. The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

[0158] A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. That is, the nucleic acids can be combined with constitutive, tissue-preferred, or other promoters for expression in the host cell of interest. In particular, for overexpression, a plant promoter fragment may be employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter (WO 99/43838 and U.S. Patent No. 6,072,050); the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell*, 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those disclosed in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142, and the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes known to those of skill. Such genes include for example, *ACT11* from *Arabidopsis* (Huang *et al.* *Plant Mol. Biol.* 33:125-139 (1996)), *Cat3* from *Arabidopsis* (GenBank No. U43147, Zhong *et al.*, *Mol. Gen. Genet.* 251:196-203 (1996)), the gene encoding stearyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe *et al.* *Plant Physiol.* 104:1167-1176 (1994)), *GPc1* from maize (GenBank No. X15596, Martinez *et al.* *J. Mol. Biol.* 208:551-565 (1989)), and *Gpc2* from maize (GenBank No. U45855, Manjunath *et al.*, *Plant Mol. Biol.* 33:97-112 (1997)).

[0159] Alternatively, the plant promoter may direct expression of chitinase nucleic acids in a specific tissue, organ or cell type (*i.e.*, tissue-specific promoters) or may be otherwise under more precise environmental or developmental control (for example, inducible promoters). Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; *e.g.*, PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, *etc.* See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. See also, WO 99/43819, which is herein incorporated by reference. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence of light, or spraying with chemicals/hormones. Tissue-

specific promoters can be inducible. Similarly, tissue-specific promoters may only promote transcription within a certain time frame or developmental stage within that tissue. Other tissue specific promoters may be active throughout the life cycle of a particular tissue. One of skill will recognize that a tissue-specific promoter may drive expression of operably linked sequences in tissues other than the target tissue. Thus, as used herein, a tissue-specific promoter is one that drives expression preferentially in the target tissue or cell type, but may also lead to some expression in other tissues as well.

[0160] A number of tissue-specific promoters can also be used in the invention. With the appropriate promoter, any organ can be targeted, such as shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit. For instance, promoters that direct expression of nucleic acids in leaves, roots or flowers are useful for enhancing resistance to pests that infect those organs. For expression of a chitinase polynucleotide in the aerial vegetative organs of a plant, photosynthetic organ-specific promoters, such as the *RBCS* promoter (Khoudi, *et al.*, *Gene* 197:343, 1997), can be used. Root-specific expression of chitinase polynucleotides can be achieved under the control of a root-specific promoter, for example, from the *ANRI* gene (Zhang & Forde, *Science*, 279:407, 1998) and Keller, *et al.*, *The Plant Cell* 3(10):1051-1061 (1991), which describes a root-specific control element in the GRP 1.8 gene of French bean. Any strong, constitutive promoters, such as the CaMV 35S promoter, can be used for the expression of chitinase polynucleotides throughout the plant.

[0161] Of particular interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200).

[0162] Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the invention. Such wound-

inducible promoters include the potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996) *Nature Biotechnology* 14:494-498); *wun1* and *wun2*, US Patent No. 5,428,148; *win1* and *win2* (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); systemin (McGurl *et al.* (1992) *Science* 225:1570-1573); WIP1 (Rohrmeier *et al.* (1993) *Plant Mol. Biol.* 22:783-792 and Eckelkamp *et al.* (1993) *FEBS Letters* 323:73-76); MPI gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like, herein incorporated by reference.

[0163] Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners; the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides; and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

[0164] Tissue-preferred promoters can be utilized to target enhanced chitinase expression within a particular plant tissue. Tissue-preferred promoters include those disclosed in Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen. Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

[0165] Leaf-specific promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.*

(1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

[0166] Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, which discloses two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa*. The promoters of these genes were linked to a β -glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) (*EMBO J.* 8(2): 343-350) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, which is an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene. The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); the ZRP2 promoter (U.S. Patent No. 5,633,363); the IFS1 promoter (U.S. Patent Application Serial No. 10/104,706) and the rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

[0167] "Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters

include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase); and celA (cellulose synthase) (see U.S. Patent No. 6,225,529, herein incorporated by reference). Gama-zein is a preferred endosperm-specific promoter. Glob-1 is a preferred embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β -phaseolin, napin, β -conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

[0168] The method of transformation/transfection is not critical to the instant invention. Various methods of transformation or transfection are currently available. As newer methods are available to transform crops or other host cells they may be directly applied to the present invention. Accordingly, a wide variety of methods have been developed to insert a DNA sequence into the genome of a host cell to obtain the transcription and/or translation of the sequence to effect phenotypic changes in the organism. Thus, any method, which provides for effective transformation/transfection may be employed with the nucleotide sequences of the present invention.

[0169] Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.*, monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (U.S. Pat Nos. 5,563,055 and 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, Eds., Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926), and Lec1 transformation (WO 00/28058). See also, Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988)

Biotechnology 6:559-563 (maize); U.S. Patent Nos. 5,240,855, 5,322,783 and 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature* (London) 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

[0170] The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrids having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that constitutive expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure constitutive expression of the desired phenotypic characteristic has been achieved.

[0171] The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*),

sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

[0172] Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), more preferably corn and soybean plants, yet more preferably corn plants.

[0173] Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli*; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang *et al.* (1977) *Nature* 198:1056), the tryptophan (trp) promoter system (Goeddel *et al.* (1980) *Nucleic Acids Res.* 8:4057), and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake *et al.* (1981) *Nature* 292:128). Examples of selection markers for *E. coli* include, for example, genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

[0174] The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for

expressing a protein of the present invention are available using *Bacillus sp.* (Palva *et al.* (1983) *Gene* 22:229-235 and Mosbach *et al.* (1983) *Nature* 302:543-545) and *Salmonella*.

[0175] A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a polynucleotide of the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra*, are employed as expression systems for the production of the proteins of the instant invention.

[0176] Synthesis of heterologous nucleotide sequences in yeast is well known. Sherman, F., *et al.* (1982) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, is a well recognized work describing the various methods available to produce a protein in yeast. Two widely utilized yeasts for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (*e.g.*, Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, an origin of replication, termination sequences and the like, as desired.

[0177] A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysate. The monitoring of the purification process can be accomplished by using Western blot techniques, radioimmunoassay or other standard immunoassay techniques.

[0178] The sequences of the present invention can also be ligated into various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Illustrative cell cultures useful for the production of these peptides are mammalian cells. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (*e.g.* the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.* (1986) *Immunol. Rev.* 89:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.*, an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection.

[0179] Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae,

silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See, Schneider, *J. Embryol. Exp. Morphol.* 27:353-365 (1987).

[0180] As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.* (1983) *J. Virol.* 45:773-781). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. See, Saveria-Campo, M., (1985) Bovine Papilloma Virus DNA a Eukaryotic Cloning Vector in *DNA Cloning Vol. II A Practical Approach*, D.M. Glover, Ed., IRL Press, Arlington, Virginia pp. 213-238.

[0181] Animal and lower eukaryotic (*e.g.*, yeast) host cells are competent or rendered competent for transfection by various means. There are several well-known methods of introducing DNA into animal cells. These include, but are not limited to: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing a DNA of interest, treatment of the recipient cells with liposomes containing a DNA of interest, DEAE dextrin, electroporation, biolistics, and micro-injection of a DNA of interest directly into the cells. The transfected cells are cultured by means well known in the art. See, Kuchler, R.J. (1997) *Biochemical Methods in Cell Culture and Virology*, Dowden, Hutchinson and Ross, Inc.

[0182] It is recognized that antisense constructions complementary to at least a portion of the messenger RNA (mRNA) for the chitinase sequences of the present invention can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

[0183] The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide

sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

[0184] In some embodiments, the content and/or composition of polypeptides of the present invention in a plant, may be modulated by altering, *In vivo* or *in vitro*, the promoter of the nucleotide sequence to up- or down-regulate expression. For instance, an isolated nucleic acid comprising a promoter sequence operably linked to a polynucleotide of the present invention is transfected into a plant cell. Subsequently, a plant cell comprising the promoter operably linked to the polynucleotide of the present invention is selected for by means known to those of skill in the art such as, but not limited to, Southern blot, DNA sequencing, or PCR analysis using primers specific to the promoter and to the polynucleotide of the present invention and detecting amplicons produced therefrom. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or composition of polypeptides of the present invention in the plant. Plant forming conditions are well known in the art.

[0185] In general, the concentration or composition of the polypeptides of the present invention is increased or decreased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% relative to a native control plant, plant part, or cell lacking the aforementioned recombinant expression cassette. Accordingly, modulation in the present invention may occur during and/or subsequent to growth of the plant to the desired stage of development. Accordingly, modulating nucleic acid expression temporally and/or in particular tissues can be controlled by employing the appropriate promoter operably linked to a polynucleotide of the present invention in, for example, sense or antisense orientation as discussed in greater detail, *supra*. Induction of the expression of a polynucleotide of the present invention can also be controlled by exogenous administration of an effective amount of inducing compound. Inducible promoters and inducing compounds, which activate expression from these promoters, are well known in the art. In preferred embodiments, the polypeptides of the present invention are modulated in monocots, particularly maize.

[0186] One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual

crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[0187] Using known procedures one of skill can screen for plants of the invention by detecting the increase or decrease of chitinase mRNA or protein in transgenic plants. Methods for detecting and quantitation of mRNAs or proteins are well known in the art.

[0188] A variety of assays can be used to determine whether a particular polypeptide has chitinase activity. Typically, activity of a chitinase candidate is compared to a negative control (*i.e.*, a sample that comprises no proteins with chitinase activity or that comprise the reagents of the sample without any other proteins). As an additional control, a candidate polypeptide's chitinase activity can be compared to chitinases such as SEQ ID NOS: 1 and 2 to identify candidates with improved enzymatic activity relative to the chitinases of SEQ ID NO: 1 and 2.

[0189] One simple test includes assaying for the ability of a polypeptide to hydrolyze carboxymethyl-chitin-remazol brilliant violet. Carboxymethyl-chitin-remazol brilliant violet is prepared according to Wirth and Wolf, *supra*. Briefly, chitin (for example, crab shell chitin (practical grade) is suspended in a base (such as, sodium hydroxide), shaken and the filtered. The dry chitin cake (retentate) is resuspended in a solution of acid in alcohol (for example, 12% chloroacetic acid in isopropanol, shaken and filtered again. The retentate is then washed twice in isopropanol and dissolved in water. The pH of the CM-chitin solution is then adjusted to about 7.

[0190] Dye can be linked to the chitin as follows. Carboxymethyl chitin (*e.g.*, 1 liter) can be heated to about 50° C and remazol brilliant violet (Sigma) (*e.g.*, 5g) is added under constant stirring. Sodium sulfate (*e.g.*, 100g) is then added in small amounts, followed by trisodium phosphate (dodecahydrate) (*e.g.*, 7.8g). After further stirring (*e.g.*, at 50° C), the CM-chitin-RBV is dialyzed against water and autoclaved.

[0191] The prepared carboxymethyl-chitin-remazol brilliant violet can be used to monitor hydrolysis as follows. Typically a purified polypeptide, crude bacterial or yeast lysate, culture supernatant or crude plant lysate containing the candidate polypeptide is added to a buffered solution (*e.g.*, 20 mM sodium acetate, pH 5.5). Following an incubation period, non-hydrolyzed chitin is precipitated with hydrochloric acid, and chitin hydrolysis is estimated by measuring the absorbance of the soluble fraction at 550 nm.

[0192] An alternate screen involves measuring the endochitinase activity of candidate polypeptides on colloidal chitin. *See*, Reissig, *et al. J. Biol. Chem.* 217:959-966 (1955); Legrand, M., *et al., Proc. Natl. Acad. Sci USA* 84:6750-6754 (1987).

[0193] Also contemplated are antipathogenic assays directed at nematode pathogens. Such assays are known to the skilled artisan, and may include assays directed at specific characteristics of nematode pathogen infections, such as assays directed at nematode feeding site formation. Such assays include those disclosed in U.S. Patent Nos. 6,008,436; and 6,252,138; herein incorporated by reference.

[0194] The present invention provides for methods of enhancing plant resistance to nematode and fungal pathogens and insects by expressing chitinase polynucleotides and/or polypeptides in plants. Such methods find use in agriculture particularly in limiting the impact of plant pathogens on crop plants. While the choice of promoter will depend on the desired timing and location of expression of the anti-pathogenic nucleotide sequences, particular promoters include constitutive and pathogen-inducible promoters. Accordingly, transformed plants, plant cells, plant tissues and seeds thereof are provided. The antifungal activity of certain chitinase proteins has been described previously. See, e.g., U.S. Patent Nos. 6,087,560; 5,993,808; 5,633,450; and 5,554,521. For example, in some embodiments, the chitinase polypeptides of the invention can be incorporated into and expressed by the tissues of a susceptible plant so that in the course of infecting the plant, the anti-fungal amounts of the selected chitinase come in contact with the invading hyphae of the invading fungus.

[0195] Enhanced resistance to any fungal pathogen is contemplated, including fungal pests such as species of *Fusarium*, *Sclerotinia*, *Botrytis*, *Cercospora*, *Gibberella*, *Oidium*, *Phytophthora*, *Sephoria*, *Verticillium*, *Alternaria*, *Cladisporium*, *Rhizoctonia*, *Ustilago*, or *Puccinia*. Specific fungal pathogens for the major crops include: Soybeans: *Phytophthora megasperma* fsp. *glycinea*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *sojae* (*Phomopsis sojae*), *Diaporthe phaseolorum* var. *caulivora*, *Sclerotium rolfsii*, *Cercospora kikuchii*, *Cercospora sojae*, *Peronospora manshurica*, *Colletotrichum dematium* (*Colletotrichum truncatum*), *Corynespora cassiicola*, *Septoria glycines*, *Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. *glycinea*, *Xanthomonas campestris* p.v. *phaseoli*, *Microsphaera diffusa*, *Fusarium semitectum*, *Phialophora gregata*, *Glomerella glycines*, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, *Heterodera glycines*, *Fusarium solani*; Canola: *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Mycosphaerella brassicicola*, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum*, *Alternaria alternata*; Alfalfa: *Clavibacter michiganese* subsp. *insidiosum*, *Pythium ultimum*, *Pythium irregulare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var.

medicaginis, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrochila medicaginis*, *Fusarium*, *Xanthomonas campestris* p.v. *alfalfae*, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*; Wheat: *Pseudomonas syringae* p.v. *atrofaciens*, *Urocystis agropyri*, *Xanthomonas campestris* p.v. *translucens*, *Pseudomonas syringae* p.v. *syringae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Collotetrachum graminicola*, *Erysiphe graminis* f.sp. *tritici*, *Puccinia graminis* f.sp. *tritici*, *Puccinia recondita* f.sp. *tritici*, *Puccinia striiformis*, *Pyrenophora tritici-repentis*, *Septoria nodorum*, *Septoria tritici*, *Septoria avenae*, *Pseudocercospora herpotrichoides*, *Rhizoctonia solani*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis* var. *tritici*, *Pythium aphanidermatum*, *Pythium arrhenomanes*, *Pythium ultimum*, *Bipolaris sorokiniana*, *Claviceps purpurea*, *Tilletia tritici*, *Tilletia laevis*, *Tilletia indica*, *Rhizoctonia solani*, *Pythium graminicola*; Sunflower: *Plasmophora halstedii*, *Sclerotinia sclerotiorum*, *Aster Yellows*, *Septoria helianthi*, *Phomopsis helianthi*, *Alternaria helianthi*, *Alternaria zinniae*, *Botrytis cinerea*, *Phoma macdonaldii*, *Macrophomina phaseolina*, *Erysiphe cichoracearum*, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus stolonifer*, *Puccinia helianthi*, *Verticillium dahliae*, *Erwinia carotovorum* pv. *carotovora*, *Cephalosporium acremonium*, *Phytophthora cryptogea*, *Albugo tragopogonis*; Corn: *Fusarium moniliforme* var. *subglutinans*, *Erwinia stewartii*, *Fusarium moniliforme*, *Gibberella zeae* (*Fusarium graminearum*), *Stenocarpella maydi* (*Diplodia maydis*), *Pythium irregulare*, *Pythium debaryanum*, *Pythium graminicola*, *Pythium splendens*, *Pythium ultimum*, *Pythium aphanidermatum*, *Aspergillus flavus*, *Bipolaris maydis* O, T (*Cochliobolus heterostrophus*), *Helminthosporium carbonum* I, II & III (*Cochliobolus carbonum*), *Exserohilum turcicum* I, II & III, *Helminthosporium pedicellatum*, *Physoderma maydis*, *Phyllosticta maydis*, *Kabatiella maydis*, *Cercospora sorghi*, *Ustilago maydis*, *Puccinia sorghi*, *Puccinia polysora*, *Macrophomina phaseolina*, *Penicillium oxalicum*, *Nigrospora oryzae*, *Cladosporium herbarum*, *Curvularia lunata*, *Curvularia inaequalis*, *Curvularia pallescens*, *Clavibacter michiganense* subsp. *nebraskense*, *Trichoderma viride*, *Claviceps sorghi*, *Pseudomonas avenae*, *Erwinia chrysanthemi* pv. *zea*, *Erwinia carotovora*, *Corn stunt Spiroplasma*, *Diplodia macrospora*, *Sclerophthora macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora maydis*, *Peronosclerospora sacchari*, *Sphacelotheca reiliana*, *Physopella zeae*, *Cephalosporium maydis*, *Cephalosporium acremonium*; Sorghum: *Exserohilum turcicum*, *Colletotrichum graminicola* (*Glomerella graminicola*), *Cercospora sorghi*, *Gloeocercospora sorghi*, *Ascochyta sorghina*, *Pseudomonas syringae* p.v. *syringae*, *Xanthomonas campestris* p.v. *holcicola*, *Pseudomonas andropogonis*, *Puccinia purpurea*, *Macrophomina phaseolina*, *Perconia circinata*, *Fusarium moniliforme*, *Alternaria alternata*, *Bipolaris sorghicola*, *Helminthosporium sorghicola*, *Curvularia lunata*, *Phoma insidiosa*, *Pseudomonas avenae*

(*Pseudomonas alboprecipitans*), *Ramulispora sorghi*, *Ramulispora sorghicola*, *Phyllachara sacchari*, *Sporisorium redianum* (*Sphacelotheca reiliana*), *Sphacelotheca cruenta*, *Sporisorium sorghi*, *Claviceps sorghi*, *Rhizoctonia solani*, *Acremonium strictum*, *Sclerophthora macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Sclerospora graminicola*, *Fusarium graminearum*, *Fusarium oxysporum*, *Pythium arrhenomanes*, *Pythium graminicola*, etc. Other fungal pathogens are described in, e.g., Agrios, PLANT PATHOLOGY (1988).

[0196] “Nematodes,” as defined herein, refers to parasitic nematodes such as cyst, root knot, and lesion nematodes, including *Heterodera* spp, *Meloidogyne* spp., and *Globodera* spp.; particularly members of the cyst nematodes, including, but not limited to, *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode); *Heterodera avenae* (cereal cyst nematode); *Globodera rostochiensis* and *Globodera pallida* (potato cyst nematodes).

[0197] Plants of interest that are susceptible to diseases caused by nematodes, and the corresponding nematodes of interest include: alfalfa: *Ditylenchus dipsaci*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Meloidogyne javanica*, *Pratylenchus* spp., *Paratylenchus* spp., *Xiphinema* spp.; banana: *Radopholus similis*, *Helicotylenchus multicinctus*, *Meloidogyne incognita*, *M. arenaria*, *M. javanica*, *Pratylenchus coffeae*, *Rotylenchulus reniformis*; beans & peas: *Meloidogyne* spp., *Heterodera* spp., *Belonolaimus* spp., *Helicotylenchus* spp., *Rotylenchulus reniformis*, *Paratrichodorus anemones*, *Trichodorus* spp.; cassava: *Rotylenchulus reniformis*, *Meloidogyne* spp.; cereals: *Anguina tritici* (Emmer, rye, spelt wheat), *Bidera avenae* (oat, wheat), *Ditylenchus dipsaci* (rye, oat), *Subanguina radiculicola* (oat, barley, wheat, rye), *Meloidogyne naasi* (barley, wheat, rye), *Pratylenchus* spp. (oat, wheat, barley, rye), *Paratylenchus* spp. (wheat), *Tylenchorhynchus* spp. (wheat, oat); chickpea: *Heterodera cajani*, *Rotylenchulus reniformis*, *Hoplolaimus seinhorsti*, *Meloidogyne* spp., *Pratylenchus* spp.; citrus: *Tylenchulus semipenetrans*, *Radopholus similis*, *Radopholus citrophilus* (Florida only), *Hemicycliophora arenaria*, *Pratylenchus* spp., *Meloidogyne* spp., *Bolonolaimus longicaudatus* (Florida only), *Trichodorus*, *Paratrichodorus*, *Xiphinema* spp.; clover: *Meloidogyne* spp., *Heterodera trifolii*; coconut: *Rhadinaphelenchus cocophilus*; coffee: *Meloidogyne incognita* (most important in Brazil), *M. exigua* (widespread), *Pratylenchus coffeae*, *Pratylenchus brachyurus*, *Radopholus similis*, *Rotylenchulus reniformis*, *Helicotylenchus* spp.; corn: *Pratylenchus* spp., *Paratrichodorus minor*, *Longidorus* spp., *Hoplolaimus columbus*; cotton: *Meloidogyne incognita*, *Belonolaimus longicaudatus*, *Rotylenchulus reniformis*, *Hoplolaimus galeatus*, *Pratylenchus* spp., *Tylenchorhynchus* spp., *Paratrichodorus minor*, grapes: *Xiphinema* spp., *Pratylenchus vulnus*, *Meloidogyne* spp., *Tylenchulus semipenetrans*, *Rotylenchulus reniformis*; grasses: *Pratylenchus* spp., *Longidorus* spp., *Paratrichodorus christiei*, *Xiphinema* spp., *Ditylenchus* spp.; peanut:

Pratylenchus spp., *Meloidogyne hapla.*, *Meloidogyne arenaria*, *Criconemella* spp., *Belonolaimus longicaudatus* (in Eastern United States); pigeonpea: *Heterodera cajani*, *Rotylenchulus reniformis*, *Hoplolaimus seinhorsti*, *Meloidogyne* spp., *Pratylenchus* spp.; pineapple: *Paratrichodorus christiei*, *Criconemella* spp., *Meloidogyne* spp., *Rotylenchulus reniformis*, *Helicotylenchus* spp., *Pratylenchus* spp., *Paratylenchus* spp.; potato: *Globodera rostochiensis*, *Globodera pallida*, *Meloidogyne* spp., *Pratylenchus* spp., *Trichodorus primitivus*, *Ditylenchus* spp., *Paratrichodorus* spp., *Nacobbus aberrans*; rice: *Aphelenchoides besseyi*, *Ditylenchus angustus*, *Hirschmanniella* spp., *Heterodera oryzae*, *Meloidogyne* spp.; small fruits: *Meloidogyne* spp., *Pratylenchus* spp., *Xiphinema* spp., *Longidorus* spp., *Paratrichodorus christiei*, *Aphelenchoides* spp. (strawberry); soybean: *Heterodera glycines*, *Meloidogyne incognita*, *Meloidogyne javanica*, *Belonolaimus* spp., *Hoplolaimus columbus*; sugar beet: *Heterodera schachtii*, *Ditylenchus dipsaci*, *Meloidogyne* spp., *Nacobbus aberrans*, *Trichodorus* spp., *Longidorus* spp., *Paratrichodorus* spp.; sugar cane: *Meloidogyne* spp., *Pratylenchus* spp., *Radopholus* spp., *Heterodera* spp., *Hoplolaimus* spp., *Helicotylenchus* spp., *Scutellonema* spp., *Belonolaimus* spp., *Tylenchorhynchus* spp., *Xiphinema* spp., *Longidorus* spp., *Paratrichodorus* spp.; tea: *Meloidogyne* spp., *Pratylenchus* spp., *Radopholus similis*, *Hemicriconemoides kanayaensis*, *Helicotylenchus* spp., *Paratylenchus curvatus*; tobacco: *Meloidogyne* spp., *Pratylenchus* spp., *Tylenchorhynchus claytoni*, *Globodera tabacum*, *Trichodorus* spp., *Xiphinema americanum*, *Ditylenchus dipsaci* (Europe only), *Paratrichodorus* spp.; tomato: *Pratylenchus* spp., *Meloidogyne* spp.; tree fruits: *Pratylenchus* spp. (apple, pear, stone fruits), *Paratylenchus* spp. (apple, pear), *Xiphinema* spp. (pear, cherry, peach), *Cacopaurus pestis* (walnut), *Meloidogyne* spp. (stone fruits, apple, etc.), *Longidorus* spp. (cherry), *Criconemella* spp. (peach), and *Tylenchulus* spp. (olive).

[0198] Enhanced resistance to fungal and nematode pathogens is generally achieved by introducing into a plant, or tissue or cell thereof, a structural gene encoding a chitinase of the invention, operably linked to plant regulatory sequences which cause expression of the chitinase gene in the plant.

[0199] As an alternative to expressing the polypeptides of the invention in plant cells, the presentation of the polypeptides can be made by formulating the polypeptide into an agricultural composition that is applied to the plant. In particular, the proteins of the invention can be formulated with an acceptable carrier into a pesticidal composition(s) for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granulae, a wettable powder, and an emulsifiable concentrate, an aerosol, an impregnated granule, an adjuvant, a cotable paste, and also encapsulations in, for example, polymer substances. Thus, presentation of the agricultural

composition may be achieved by external application either directly or in the vicinity of the plants or plant parts. The agricultural compositions may be applied to the environment of the fungal, nematode and insect pest(s), *e.g.*, plants, soil or water, by spraying, dusting, sprinkling, or the like.

[0200] The present invention further contemplates using recombinant hosts, including microbial hosts and insect viruses, transformed with a gene encoding the chitinase polypeptides of the invention and applied on or near a selected plant or plant part susceptible to attack by a target pest. The hosts may be capable of colonizing a plant tissue susceptible to infestation or of being applied as dead or non-viable cells containing the chitinase. Microbial hosts of particular interest will be the prokaryotes and the lower eukaryotes, such as non-chitin-containing fungi (*e.g.*, oomycetes). In some embodiments, the microbial host secretes the chitinase into their surrounding environment so as to contact a fungal cell or nematode.

[0201] Examples of prokaryotes, both Gram-negative and -positive, that are potentially useful for expressing chitinases include *Enterobacteriaceae*, such as *Escherichia*; *Bacillaceae*; *Rhizoboceae*, such as *Rhizobium* and *Rhizobacter*; *Spirillaceae* (such as photobacterium), *Zymomonas*, *Serratia*, *Aeromonas*, *Vibrio*, *Desulfovibrio*, *Spirillum*; *Lactobacillaceae*; *Pseudomonadaceae* (such as *Pseudomonas* and *Acetobacter*); *Azotobacteraceae* and *Nitrobacteraceae*.

[0202] Bacteria, and particularly *rhizobacteria*, modified in accordance with the present invention and grown to sufficient proportions, *e.g.*, by fermentation, can be used to combat chitin-containing soil pathogens by application of the bacteria to soil, seeds, vegetative plant parts or irrigation water. For example, mucolytic bacteria created in accordance with the invention can be used in such ways to attack or inhibit fungi. The microbial host can be applied in various formulations containing agronomically acceptable adjuvants or carriers in dosages and concentrations chosen to maximize the beneficial effect of the *rhizobacteria*.

[0203] For application to soil, to soil mixes, or to artificial plant growth media, the microbial host may be applied as a powder or granule in a suitable carrier. Alternatively, the microbial host may be applied as a suspension or dispersion, *e.g.*, as an aqueous suspension with a suitable protectant such as methylcellulose, dextran, dextrin, alginate, magnesium silicate. The microbial host may also be applied as a wettable powder.

[0204] For application to seeds, the microbial host may be applied as part of a seed coating composition, for instance mixed with xanthan gum, magnesium silicate, methylcellulose, gum arabic, polyvinyl pyrrolidone, dextrans or dextrans. In addition, small amounts of partially

hydrolyzed chitin may be added to the pelleting mix, dust granule, suspension, or wettable powder to enhance chitinase production. *See, generally, Suslow et al., Phytopathology* 72:199-206 (1982); and Kloepper *et al., Phytopathology* 71:590-592 (1981), for a discussion of *rhizobacteria* and seed coating compositions.

[0205] Bacteria expressing a chitinase in accordance with the present invention may also be applied to the above-ground surface of a plant, *e.g.*, the leaf or stem surface, either to permit the modified bacteria to travel or spread to the roots or to inhibit chitinase-sensitive pathogens which may be present on blossoms or plant surfaces, for instance, fungal pathogens such as *Botrytis*, *Monilinia*, *Alternaria*, and *Cercospora*. Blossoms of *Prunus* sp., in particular, provide an ideal environment for the growth of epiphytic bacteria, *e.g.*, *Pseudomonas syringae* or *Erwinia herbicola*, which have the ability to produce inhibitory levels of chitinase.

[0206] The method of the invention can also be used for introduction of chitinase genes into species of *Rhizobium* which enter into a nitrogen fixing symbiosis within the nodules of leguminous plants. The nodules are frequently the point of entry of pathogenic fungi and nematodes.

[0207] The recombinant host may be formulated in a variety of ways. It may be employed in wettable powders, granules or dusts, or by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other insecticidal additives, surfactants, and bacterial nutrients or other agents to enhance growth or stabilize bacterial cells. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers. In general, inoculants can be applied at any time during plant growth. Inoculation of large fields can be accomplished most effectively by spraying.

[0208] Plants with enhanced resistance can be selected in many ways known to those of skill in the art. For example, to assess resistance to fungal and nematode attack, transgenic plants expressing the polypeptides of the invention are exposed to a fungal or nematode pathogen to which the wild type plant is susceptible. In some cases, for instance, the soil is infested with fungal spores or nematodes. The plants are then monitored over a time period sufficient for pathogenesis by the fungus or nematode (*e.g.*, one to four weeks). Monitoring of plants includes observation of viability, height, root mass and leaf area.

[0209] The polypeptides of the invention may be used alone or in combination with other proteins or agents to control different fungal and nematode pathogens. For example, chitinase can be combined with β -1,3 glucanase and/or ribosome inactivating protein (RIP). See, e.g., Jach, *Plant J.* 8(1):97-109 (1995). Other antimicrobial components that can be combined with the chitinase of the invention include those discussed in Lamb, *et al. Biotechnology (N.Y.)* 10(11):1436-45 (1992).

[0210] Other examples of proteins that may be used in combination with antifungal/antinematocidal proteins according to the invention include, but are not limited to, β -1,3-glucanases and other chitinases such as those obtainable from barley (Swegle M. *et al*, 1989, *Plant Mol. Biol.* 12, 403-412; Balance G. M. *et al*, 1976, *Can. J. Plant Sci.* 56, 459-466; Hoj P. B. *et al*, 1988, *FEBS Lett.* 230, 67-71; Hoj P. B. *et al*, 1989, *Plant Mol. Biol.* 13, 31-42 1989), bean (Boller T. *et al*, 1983, *Planta* 157, 22-31; Broglie K. E. *et al.* 1986, *Proc. Natl. Acad. Sci. USA* 83, 6820-6824; 1988 *Planta* 174, 364-372); Mauch F. & Staehelin L. A., 1989, *Plant-Cell* 1, 447-457); cucumber (Metraux J. P. & Boller T. (1986), *Physiol. Mol. Plant Pathol.* 28, 161-169); leek (Spanu P. *et al*, 1989, *Planta* 177, 447-455); maize (Nasser W. *et al*, 1988, *Plant Mol. Biol.* 11, 529-538), oat (Fink W. *et al*, 1988, *Plant Physiol.* 88, 270-275), pea (Mauch F. *et al* 1984, *Plant Physiol.* 76, 607-611; Mauch F. *et al*, 1988, *Plant Physiol.* 87, 325-333), poplar (Parsons, T. J. *et al*, 1989, *Proc. Natl. Acad. Sci. USA.* 86, 7895-7899), potato (Gaynor J. J. 1988, *Nucl. Acids Res.* 16, 5210; Kombrink E. *et al* 1988, *Proc. Natl. Acad. Sci. USA* 85, 782-786; Laflamme D. and Roxby R., 1989, *Plant Mol. Biol.* 13, 249-250), tobacco (e.g. Legrand M. *et al* 1987, *Proc. Natl. Acad. Sci. USA* 84, 6750-6754; Shinshi H. *et al.* 1987, *Proc. Natl. Acad. Sci. USA* 84, 89-93), tomato (Joosten M. H. A. & De Wit P. J. G. M. 1989, *Plant Physiol.* 89, 945-951), wheat (Molano J. *et al*, 1979, *J. Biol. Chem.* 254, 4901-4907), and the like.

[0211] The following examples are offered by way of illustration and not by way of limitation.

Examples

Example 1

Cloning and Protein Expression of the Polynucleotides of the Invention

[0212] Genes, referred to as parent genes, were amplified by PCR, with flanking regions containing restriction sites *Cla* I at the 5'-end and *Xba* I followed by a stop site at the 3'-end. The amplified sequences were ligated, in frame with the α --factor signal sequence, to the *E.*

coli-Pichia pastoris shuttle vector pPICZ α -C (Invitrogen) in which the DNA fragment comprised between Cla I and Xba I had been deleted. Cloning of the constructed plasmid in the *E. coli* strain Top 10 F' (Invitrogen) was performed according to the manufacturer's instructions.

[0213] A library of nucleic acids (in some cases comprising a polynucleotide encoding a histidine tag) related to the parent gene sequences was obtained. The polynucleotides of the library were then ligated into pPICZ α -C, transformed into *E. coli* and the library was then amplified on LSLB agar plates supplemented with 25 μ M Zeocin.

[0214] Plasmid DNA from the entire library was extracted, linearized at a unique Pme I restriction site, and transformed by multiple electroporation events into the *Pichia pastoris* strain KM71. After selection of *Pichia pastoris* transformants on YPDS agar plates containing 100 μ M Zeocin, expression of the chitinases was induced in liquid format. High throughput expression required initial growth of the selected *Pichia pastoris* clones in 400 to 800 μ l of BMGY medium, supplemented with a glass bead (biomass production). Growth was performed at 30°C and 80% humidity under vigorous shaking. After 2-3 days of biomass production, the *Pichia* cultures were centrifuged and resuspended in minimal medium (BMMH) for chitinase expression. Maximum expression was obtained after four days of culture under conditions similar to those used for biomass production.

Example 2

Identification of Chitinase Clones with Improved Chitinase Activity

[0215] In general, the chitinases with improved activity were identified as follows. Proteins secreted to the culture medium were assayed for their ability to hydrolyze chitin. The clones with the best hydrolytic activity against carboxymethyl-chitin-remazol brilliant violet (CM-chitin-RBV) were selected. DNA from the most active clones was isolated. Selected chitinase clones were sequenced by using PCR primers specific to regions flanking the genes.

[0216] Two colorimetric methods were used to determine the chitinolytic activity of the clones: one based on the hydrolysis of the soluble, dye-labelled substrate carboxymethyl-chitin-remazol brilliant violet (CM-chitin-RBV) (Wirth and Wolf *J. Microbiol. Methods* 12:197-205 (1990)), and the second by measuring the endochitinase activity of the enzymes on colloidal chitin (Reissig, *et al. J. Biol. Chem.* 217:959-966 (1955); Legrand, *et al. Proc. Natl. Acad. Sci USA* 84:6750-6754 (1987)).

[0217] Selection of active clones was performed in 96-well microtiter plates by adding 5 μ l of *Pichia* culture supernatant to wells containing the substrate CM-chitin-RBV in 20 mM sodium acetate buffer, pH 5.5. After a 30 min incubation at 37°C, acid-insoluble chitin was precipitated with HCl and the amount of chitin hydrolyzed by the enzymatic treatment was estimated by measuring the absorbance at 550 nm of the supernatant.

[0218] Clones that tested positive for enzyme activity were re-arrayed into microtiter plates, expressed again and re-tested. The clones with the best activity were then further characterized.

[0219] The clones with the best activity were grown and expressed in bulk. The so produced chitinases, which represented ~90% of the protein content of the *Pichia* culture supernatants, were concentrated 100 to 200-fold with centrifugal concentration devices and dialyzed over night against reaction buffer (20 mM sodium acetate, pH 5.5). Chitinases normalized for protein concentration were used in endochitinase and in CM-chitin-RBV hydrolysis assays under substrate-saturating conditions. Under such conditions, the chitinolytic reactions were linear with respect to enzyme concentration. The activity of the improved chitinases was expressed in multiples of the activity of the best wild-type control protein (chitinase A (SEQ ID NO: 1)). Table 2 demonstrates the results of the CM-chitin-RBV hydrolysis assays. Table 3 shows the results of the endochitinase assays.

[0220] K_m and V_{max} values were determined for the product of the clone r1B10 (SEQ ID NO: 6) using the CM-chitin-RBV hydrolysis assay. The gene product was found to have the same K_m as the expressed wild-type chitinase A (SEQ ID NO: 1). The V_{max} of the gene product was 3- to 4-fold higher than wild-type chitinase A.

[0221] Figures 2 and 3 illustrate the sequence relationship between SEQ ID NO: 1 and the improved chitinase sequences. Figure 2 illustrates the nucleotide differences between the clones and Figure 3 illustrates amino acid differences between the gene products.

Table 2
Activity of the novel chitinases as determined by the endochitinase assay

Clone	Improvement over wild-type chitinase A (SEQ ID NO: 1)
r1B6 (SEQ ID NO: 4)	3.2-fold
r1B10 (SEQ ID NO: 6)	3.6-fold
r1D4 (SEQ ID NO: 8)	3.9-fold
r2A2 (SEQ ID NO: 10)	4.3-fold
r2C2 (SEQ ID NO: 12)	3.2-fold
r2E1 (SEQ ID NO: 14)	6.6-fold
r2H2 (SEQ ID NO: 16)	4.6-fold

Table 3
Activity of the novel chitinases as determined by CM-chitin-RBV hydrolysis

Clone	Improvement over wild-type chitinase A (SEQ ID NO: 1)
r1B6 (SEQ ID NO: 4)	5.6-fold
r1B10 (SEQ ID NO: 6)	5.1-fold
r1D4 (SEQ ID NO: 8)	1.9-fold
r2A2 (SEQ ID NO: 10)	4.8-fold
r2C2 (SEQ ID NO: 12)	4.5-fold
r2E1 (SEQ ID NO: 14)	5.4-fold
r2H2 (SEQ ID NO: 16)	8.9-fold

Example 3:

Improved Antifungal Activity of a Chitinase Clone with Enhanced Chitinolytic Activity

[0222] Clones with enhanced chitinase activity were tested for their ability to prevent hyphal growth of the pathogenic fungus *Fusarium moniliforme*. For preliminary screens, secreted protein was concentrated and buffer-exchanged before being used in antifungal assays. The secreted gene product was typically 90-95% pure, according to Coomassie-stained SDS-PAGE. For more precise characterization, protein secreted into the *Pichia pastoris* culture medium was concentrated and purified before being used in the antifungal assays.

[0223] Spores of *Fusarium moniliforme* were pre-germinated in clear 96-well microtiter plates in Vogel's Minimal Medium (VMM) (Vogel, *Microb. Genet. Bull.* 13:42-43 (1956); Vogel, *Am. Nature* 98:435-446 (1964)). Chitinases expressed with a histidine tag at their C-terminus were purified over nickel-nitrilotriacetic acid-charged agarose beads (Ni-NTA

Superflow, Qiagen) according to the manufacturer's instructions. Chitinases were eluted from the Ni-NTA matrix with a phosphate buffer containing 250 mM imidazole. The imidazole-containing buffer was exchanged for 20 mM sodium acetate, pH 5.5, by dialysis or by centrifugation through centrifugal filtration devices. Purified chitinases were added to the germinated spores to final concentrations of 0, 20, 50, 100, 200 and 500 µg/ml. In some instances, 6 µg/ml of β -1,3-glucanase from *Helix pomatia* was added to the mix. After incubation at 25°C for ~45 h, the absorbance at 600 nm was recorded for each of the chitinase-containing microtiter wells. Figure 1 demonstrates the antifungal activity of the gene product r2C2 (SEQ ID NO: 12) as compared to the antifungal activity of the chitinase A (SEQ ID NO: 1). Forty percent inhibition of hyphal growth was achieved with a ~4-fold lower concentration of the r2C2 (SEQ ID NO: 12) gene product, as compared to chitinase A (SEQ ID NO: 1).

Example 4

Improved Antifungal Activity of Additional Chitinase Clones with Enhanced Chitinolytic Activity

[0224] Additional clones with enhanced chitinase activity were tested for their ability to prevent hyphal growth of the pathogenic fungus *Fusarium moniliforme*. For preliminary screens, secreted protein was concentrated and buffer-exchanged before being used in antifungal assays. The secreted gene product was typically 90-95% pure, according to Coomassie-stained SDS-PAGE. For more precise characterization, protein secreted into the *Pichia pastoris* culture medium was concentrated and purified before being used in the antifungal assays.

[0225] Spores of *Fusarium moniliforme* were pre-germinated in clear 96-well microtiter plates in Vogel's Minimal Medium (VMM) (Vogel, *Microb. Genet. Bull.* 13:42-43 (1956); Vogel, *Am. Nature* 98:435-446 (1964)). Purified chitinases were added to the germinated spores to final concentrations between 0 and 200 µg/ml. Six µg/ml of β -1,3-glucanase from *Helix pomatia* was added to the mix. After incubation at 25°C for ~45 h, the absorbance at 600 nm was recorded for each of the chitinase-containing microtiter wells. Figure 4A illustrates the average concentration of chitinase required to inhibit fungal growth by 50% and 4B demonstrates the improved antifungal activity of the gene products 4M1/1_H2 (SEQ ID NO: 24), 4M1/11_C10 (SEQ ID NO: 22), and 2M1/26_C2 (SEQ ID NO: 26) as compared to the antifungal activity of the previously identified "hit" r2C2 (SEQ ID NO: 12). Figure 5 illustrates

antifungal activity of variant 4M1/11_C10 (SEQ ID NO: 22), as compared to the wild-type protein chitinase A (SEQ ID NO: 1) (labeled “1-2SCH” in the figure) and variant “r2C2” (SEQ ID NO: 12).

[0226] Additional clones from further rounds of shuffling were tested for antifungal activity. Specifically, pores of *Fusarium moniliforme* were pre-germinated in clear 96-well microtiter plates in Vogel’s Minimal Medium (VMM) (Vogel, *Microb. Genet. Bull.* 13:42-43 (1956); Vogel, *Am. Nature* 98:435-446 (1964)). Purified chitinases were added to the germinated spores to final concentrations between 0 and 100 µg/ml. Six µg/ml of β -1,3-glucanase from *Helix pomatia* was added to the mix. After incubation at 25°C for ~45 h, the absorbance at 600 nm was recorded for each of the chitinase-containing microtiter wells. Figures 13 and 14 and Table 4 demonstrate the improved antifungal activity of gene products resulting from the fourth round of shuffling. In Table 4 are compared to the antifungal activity of the previously identified “hit” 4N1/88_F9 (SEQ ID NO: 56) and of the wild-type protein, chitinase A (SEQ ID NO: 1), also called 1-2SCH.

Table 4
Antifungal activity of improved chitinases from 4th round of shuffling.

Clone		Fold improvement over 4N1/88_F9 (SEQ ID NO: 56)	Fold improvement over chitinase A (SEQ ID NO: 1)
4Q2/10_B8 (SEQ ID NO: 76)	ID	1.39	32.0
4Q2/13_F8 (SEQ ID NO: 78)	ID	1.09	25.1
4P1/2_B5 (SEQ ID NO: 68)		1.1	25.1
4P1/7_H9 (SEQ ID NO: 70)		1.2	28.3
4Q1/3_H10 (SEQ ID NO: 72)		1.0	23.7
4Q1/5_B11 (SEQ ID NO: 74)		1.1	24.6
4R2/1_G10 (SEQ ID NO: 80)		1.1	25.1
4R2/5_H11 (SEQ ID NO: 82)		1.2	26.5
4R2/9_B2 (SEQ ID NO: 84)	ID	1.0	23.2

[0227] Purified chitinases were added to germinating spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. Activity measurements were compared to those obtained with the previously identified hit 4N1/88_F9 (SEQ ID NO: 56), and improvements relative to the wild-type clone 1-2SC (chitinase A) (SEQ ID NO: 1) were calculated. Ovalbumin and bovine serum albumin (BSA) were used as standards for protein determinations. Therefore, IC₅₀ values are expressed in ovalbumin and in BSA equivalents. Average fold improvements are described. A summary of improved hits is provided in Figure 15. Figures 16, 17, 18A-18B and 19 show alignments of DNA and protein sequences of the forth round hits. Figure 16 illustrates the nucleotide differences between the clones 4Q2/10_B8 (SEQ ID NO: 76) and 4Q2/13_F8 (SEQ ID NO: 78) and Figure 17 illustrates amino acid differences between the gene products 4Q2/10_B8 (SEQ ID NO: 76) and 4Q2/13_F8 (SEQ ID NO: 78). Figure 18A and 18B illustrate the nucleotide differences between the clones 4P1/2_B5 (SEQ ID NO: 68), 4P1/7_H9 (SEQ ID NO: 70), Q1/3_H10 (SEQ ID NO: 72), 4Q1/5_B11 (SEQ ID NO: 74), 4R2/1_G10 (SEQ ID NO: 80), 4R2/5_H11 (SEQ ID NO: 82), and 4R2/9_B2 (SEQ ID NO: 84) and Figure 19 illustrates amino acid differences between the gene products 4P1/2_B5 (SEQ ID NO: 68), 4P1/7_H9 (SEQ ID NO: 70), Q1/3_H10 (SEQ ID NO: 72), 4Q1/5_B11 (SEQ ID NO: 74), 4R2/1_G10 (SEQ ID NO: 80), 4R2/5_H11 (SEQ ID NO: 82), and 4R2/9_B2 (SEQ ID NO: 84).

Example 5

New Method for Testing the Antifungal Activity of Chitinases and the Improved Antifungal Activity of Additional Chitinase Clones with Enhanced Chitinolytic Activity

[0228] Purified chitinases were tested for their ability to prevent hyphal growth of the pathogenic fungus *Fusarium moniliforme* grown on solid agar. In classic antifungal assays, anti-fungal proteins are added to filter paper disks deposited onto agar plates used to grow the pathogenic fungi. As the fungus grows towards the filter disk, a clearing zone is produced around the disk. We have found that *Fusarium moniliforme* responds poorly to these classic antifungal assays, either because the anti-fungal protein (*i.e.*, chitinases) do not diffuse readily into the agar medium, or because *Fusarium moniliforme* hyphae can grow over a long distance without coming into contact with the medium. Therefore, we incorporated the chitinases under test directly into Vogel's Minimal Medium, VMM (Vogel, 1956, 1964), modified so as to contain only 1% sucrose, and poured the chitinase-agar medium into the wells of a 96-well microtiter plate. When the medium solidified, spores of *Fusarium moniliforme* were added to

the wells and incubated at 22°C. The effect of the chitinases incorporated into the growth medium was evaluated under an inverted microscope. Figure 8 demonstrates the effect of 100 or 300 µg of chitinase incorporated into 100 µl of solid growth medium. The wild-type chitinase, 1-2SCH (chitinase A) (SEQ ID NO: 1) is compared to the previously identified “hit” r2C2 (SEQ ID NO: 12) and “hit” 4N1/88_F9 (SEQ ID NO: 56).

[0229] Improvement of new chitinases as compared to the best wild-type clone and the previously identified hit “r2C2” (SEQ ID NO: 12) are illustrated in Table 5. The fold-improvement of a clone reflects the reduced protein concentration required to achieve similar inhibition of *Fusarium moniliforme* growth compared to “chitinase A” (SEQ ID NO: 1) and “r2C2” (SEQ ID NO: 12).

Table 5

Clone	Fold improvement over r2C2 (SEQ ID NO: 12)	Fold improvement over chitinase A (SEQ ID NO: 1)
R1AB2 (SEQ ID NO: 28)	1.2	4.6
r1AD4 (SEQ ID NO: 30)	0.7	2.8
r1AD6 (SEQ ID NO: 32)	0.6	2.3
r1AG9 (SEQ ID NO: 34)	0.9	3.5
r1AH8 (SEQ ID NO: 36)	1.6	6.2
r1AH9 (SEQ ID NO: 38)	2.0	8.0
r1BG5 (SEQ ID NO: 40)	1.0	4.1
r2C5 (SEQ ID NO: 42)	0.7	2.8

[0230] Spores of *Fusarium moniliforme* were pre-germinated in clear 96-well microtiter plates in Vogel’s Minimal Medium (VMM) (Vogel, *Microb. Genet. Bull.* 13:42-43 (1956);

Vogel, *Am. Nature* 98:435-446 (1964)). Purified chitinases were added to the germinated spores to final concentrations between 0 and 200 $\mu\text{g/ml}$. Six $\mu\text{g/ml}$ of β -1,3-glucanase from *Helix pomatia* was added to the mix. After incubation at 25°C for ~45 h, the absorbance at 600 nm was recorded for each of the chitinase-containing microtiter wells. Figure 9 and Table 5 demonstrate the improved antifungal activity of gene products resulting from the first and second rounds of shuffling: r1AB2 (SEQ ID NO: 28); r1AD4 (SEQ ID NO: 30); r1AD6 (SEQ ID NO: 36); r1AG9 (SEQ ID NO: 34); r1AH8 (SEQ ID NO: 36); r1AH9 (SEQ ID NO: 38); r1BG5 (SEQ ID NO: 40), and r2C5 (SEQ ID NO: 42). They are compared to the antifungal activity of the previously identified “hit” r2C2 (SEQ ID NO: 12) and of the wild-type protein (chitinase A) (SEQ ID NO: 1), also named 1-2SCH. Ovalbumin was used as a standard for protein determinations. Therefore, IC_{50} values are expressed in ovalbumin equivalents. If bovine serum albumin (BSA) had been used as a standard, the IC_{50} values would be reduced by a factor 4.5. In other words, the IC_{50} values would be 4.5-times lower (or better). The results expressed in “fold improvement” would remain unchanged. Results from a further selection of improved chitinases are shown in Table 6. Average fold improvements are described. The average IC_{50} for the 12 improved hits equals 23 $\mu\text{g/ml}$. Table 6 illustrates further improved chitinases. Purified chitinases were added to germinating spores of *Fusarium moniliforme* and their efficiency at inhibiting fungal growth was recorded by taking absorbance measurements at 600 nm. Activity measurements were compared to those obtained with the previously identified hit 4M1/11_C10 (SEQ ID NO: 22), and improvements relative to the wild-type clone 1-2SC (chitinase A) (SEQ ID NO: 1) were calculated.

Table 6
Antifungal activity of further improved chitinases

Clone	Fold improvement over 4M1/1_C10 (SEQ ID NO: 22)	Fold improvement over chitinase A (SEQ ID NO: 1)
4N1/11_B11 (SEQ ID NO: 62)	1.2	18
4N1/14_B3 (SEQ ID NO: 58)	1.1	17
4N1/2_H9 (SEQ ID NO: 50)	1.35	20
4N1/23_G4 (SEQ ID NO: 52)	1.5	23
4N1/30_D3 (SEQ ID NO: 64)	1.4	21
4N1/33_F4 (SEQ ID NO: 60)	1.4	21
4N1/35_G5 (SEQ ID NO: 66)	1.55	23
4N1/68_E4 (SEQ ID NO: 54)	1.25	19
4N1/75_D3 (SEQ ID NO: 48)	1.3	20
4N1/80_F8 (SEQ ID NO: 46)	1.15	17
4N1/88_F9 (SEQ ID NO: 56)	1.55	23
4N1/95_H3 (SEQ ID NO: 44)	1.3	20

[0231] A summary of improved hit is provided in Figure 10. Figures 11A to 11C and Figure 12 show alignments of nucleic acid and protein sequences (compared to clone r1AB2 (SEQ ID NO: 28)) identified in this improved set of chitinases. Figures 11A to 11C are comparative nucleotide alignments between a selection of the polynucleotides of the invention (r1AB2 (SEQ ID NO: 27), r1AD4 (SEQ ID NO: 29), r1AD6 (SEQ ID NO: 31), r1AG9 (SEQ ID NO: 33), r1AH8 (SEQ ID NO: 35), r1AH9 (SEQ ID NO: 37), r1BG5 (SEQ ID NO: 39), r2C5 (SEQ ID NO: 41), 4N1/95_H3 (SEQ ID NO: 43), 4N1/80_F8 (SEQ ID NO: 45), 4N1/75_D3 (SEQ ID NO: 47), 4N1/2_H9 (SEQ ID NO: 49), 4N1/23_G4 (SEQ ID NO: 51), 4N1/68_E4 (SEQ ID NO: 53), 4N1/88_F9 (SEQ ID NO: 55), 4N1/14_B3 (SEQ ID NO: 57), 4N1/33_F4 (SEQ ID NO: 59), 4N1/11_B11 (SEQ ID NO: 61), 4N1/30_D3 (SEQ ID NO: 63), and 4N1/35_G5 (SEQ ID NO: 65)). Figure 12 illustrates a comparative amino acid alignment between a selection of the polypeptides of the invention (r1AB2 (SEQ ID NO: 28), r1AD4 (SEQ ID NO: 30), r1AD6 (SEQ ID NO: 32), r1AG9 (SEQ ID NO: 34), r1AH8 (SEQ ID NO: 36), r1AH9 (SEQ ID NO: 38), r1BG5 (SEQ ID NO: 40), r2C5 (SEQ ID NO: 42), 4N1/95_H3 (SEQ ID NO: 44), 4N1/80_F8 (SEQ ID NO: 46), 4N1/75_D3 (SEQ ID NO: 48), 4N1/2_H9 (SEQ ID NO: 50), 4N1/23_G4 (SEQ ID NO: 52), 4N1/68_E4 (SEQ ID NO: 54), 4N1/88_F9 (SEQ ID NO: 56), 4N1/14_B3 (SEQ ID NO: 58), 4N1/33_F4 (SEQ ID NO: 60), 4N1/11_B11 (SEQ ID NO: 62), 4N1/30_D3 (SEQ ID NO: 64), and 4N1/35_G5 (SEQ ID NO: 66)).

Example 6

Anti-nematode Activity of Chitinase Clones

[0232] Purified proteins from shuffled chitinases were assayed in a typical nematode assay, setup in 96-well microtiter plates, and using *C. elegans*. Nematode eggs or juveniles at stage L1 were added to an assay buffer (10 mM Sodium acetate buffer, pH 5.5) containing or not containing chitinases. The assay mix was further supplemented with the antibiotics tetracycline and chloramphenicol, both at a concentration of 30 $\mu\text{g/ml}$. 10 μl of a saturated culture of *E. coli* OP50 were added to each well to serve as food for the nematodes. After one day, hatching of the eggs was recorded. After 6 days of incubation at 22°C, the microtiter plates were scored, by counting the number of nematodes of each stage present in the wells. The scores are: L1, L2 and L3 (successive larval stages); YA (young adult); A (adult), and E (next generation eggs). The chitinases used in this assay were r2C2 (SEQ ID NO: 12) and 4M1/11_C10 (SEQ ID NO: 22). The control contained buffer alone. An average of eight L1 larvae and 38 eggs were dispensed

into each well at time zero. All treatments were done in duplicates (I and II). Results are presented in Table 7.

Table 7

**Anti-fungal scores obtained with shuffled chitinases
r2C2 (SEQ ID NO: 12) and 4M1/11_C10 (SEQ ID NO: 22)**

Protein	Protein added $\mu\text{g/ml}$ (final)	Day 0	Day 1	Day 6	
				I	II
Control	0	~8L1s+38eggs	All eggs hatched	L4-A	L4-A
r2C2					
(SEQ ID NO: 12)	103	~8L1s+38eggs	All eggs hatched	L3-A	L3-A
r2C2					
(SEQ ID NO: 12)	258	~8L1s+38eggs	All eggs hatched	L3-A	L3-A
r2C2					
(SEQ ID NO: 12)	517	~8L1s+38eggs	All eggs hatched	<u>L1-L3</u>	L1-L4
4M1/11_C10					
(SEQ ID NO: 22)	23	~8L1s+38eggs	All eggs hatched	<u>L1-L3</u>	L1-L3
4M1/11_C10					
(SEQ ID NO: 22)	58	~8L1s+38eggs	All eggs hatched	L1-L3	L1-L3
4M1/11_C10					
(SEQ ID NO: 22)	117	~8L1s+38eggs	All eggs hatched	<u>L1-L2</u>	-

[0233] Table 7 shows that, while in the buffer control, the nematodes reached stage L4 to A, in the chitinase-treated wells; the development of the worms was slowed. These results indicate that for both, r2C2 (SEQ ID NO: 12) and 4M1/11_C10 (SEQ ID NO: 22), the inhibitory effect was concentration-dependent, with 4M1/11_C10 (SEQ ID NO: 22) being more potent than r2C2 (SEQ ID NO: 12).

[0234] The effect of heat denaturation on the activity of the chitinases is described in Table 8. Denaturation was obtained by heating the proteins to 85°C for 10 min. Two samples r2C2 (SEQ ID NO: 12) and 4M1/11_C10 (SEQ ID NO: 22) were again tested under each condition (I and II). The experiment was started with about 50 L1 larvae in each well, and the nematodes were scored at days 5 and 9. The results in Table 8 show again the concentration-dependent activity of the chitinases in slowing the development of the nematodes. Overall, denatured chitinases did not maintain their inhibitory effect, indicating that the structure and/or the enzymatic activity of the chitinases were required for their anti-nematode activity.

Table 8
Effect of heat inactivation of chitinases

Protein	Condition	[Protein] μg/ml	Day 5		Day 9	
			I	II	I	II
Control	No protein	0	YA	YA	A	A
Control	No protein	0	YA	YA	A	A
4M1/11_C10 (SEQ ID NO: 22)	native	23	L2-L3*	L1-L2	L3-YA*	L1-L2
4M1/11_C10 (SEQ ID NO: 22)	native	58	L1-L2	L1-L2	L1-L2	L1-L2
4M1/11_C10 (SEQ ID NO: 22)	native	117	L1-L2	L1-L2	L1-L2	L1-L2
4M1/11_C10 (SEQ ID NO: 22)	denatured	23	YA	YA	A	A
4M1/11_C10 (SEQ ID NO: 22)	denatured	58	YA	YA	A	A
4M1/11_C10 (SEQ ID NO: 22)	denatured	117	YA	YA	A, L1	A, L1
R2C2 (SEQ ID NO: 12)	native	52	L2-L4	L1-L3	L4-YA	A
R2C2 (SEQ ID NO: 12)	native	129	L1-L3	L1-L2	L2-L4	L1-L2
R2C2 (SEQ ID NO: 12)	native	258	L1-L2	L1-L2	L2-L4	L2-L4
R2C2 (SEQ ID NO: 12)	denatured	52	YA	YA	A	A
R2C2 (SEQ ID NO: 12)	denatured	129	L3-YA	L2-L4	L3-A	L3-A
R2C2 (SEQ ID NO: 12)	denatured	258	L1-L3	L1-L3	L1-L3	L1-L3
4M1/11_C10 (H ₂ O) (SEQ ID NO: 22)	native	58	L1-L2		L1-L2	

* Wells were contaminated

[0235] An additional experiment was performed to compare the anti-nematode effect of wild-type chitinase ZmCht1-2 (chitinase A) (SEQ ID NO: 1), r2C2 (SEQ ID NO: 12), 4M1/11_C10 (SEQ ID NO: 22) and 4N1/88_F9 (SEQ ID NO: 56). The results are shown in Table 9. The inhibitory effect of the chitinase is scored as “yes”, while no effect is scored as “no”. The results shown in Table 9 indicate that the wild-type chitinase “chitinase A” (SEQ ID NO: 1) exhibits no inhibitory effect on the development of *C. elegans*. The chitinase R2C2 (SEQ ID NO: 12) and the chitinase 4N1/88_F9 (SEQ ID NO: 56) are both inhibitory at

concentrations between 9 µg/ml and 45 µg/ml. The chitinase 4M1/11_C10 (SEQ ID NO: 22) is the most potent, requiring as little as 9 µg/ml to exhibit an anti-nematocidal effect.

Table 9
Comparison of chitinases

[Chitinase]	Chitinase			
	chitinase A (SEQ ID NO: 1)	r2C2 (SEQ ID NO: 12)	4M1/11_C10 (SEQ ID NO: 22)	4N1/88_F9 (SEQ ID NO: 56)
9 µg/ml	No	no	yes	no
9 µg/ml	No	no	yes	no
45 µg/ml	No	yes	yes	yes
45 µg/ml	No	yes	yes	yes
91 µg/ml	No	yes	yes	yes
91 µg/ml	No	yes	yes	yes
273 µg/ml	No	yes	yes	yes
273 µg/ml	No	yes	yes	yes

Example 7

Transformation and Regeneration of Transgenic Plants

[0236] Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a chitinase nucleotide sequence operably linked to a ubiquitin promoter and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as described below. Media recipes follow below.

[0237] The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

[0238] A plasmid vector comprising the chitinase nucleotide sequence operably linked to a ubiquitin promoter is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 µm (average diameter) tungsten pellets using a CaCl₂ precipitation procedure as follows:

100 µl prepared tungsten particles in water

10 µl (1 µg) DNA in Tris EDTA buffer (1 µg total DNA)

100 µl 2.5 M CaCl₂

10 µl 0.1 M spermidine

[0239] Each reagent is added sequentially to the tungsten particle suspension, which is maintained on a multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml of 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µls of 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl is spotted onto the center of each macrocarrier and allowed to dry for about 2 minutes before bombardment.

[0240] The sample plates are bombarded at manufacturers recommended levels in a particle gun commercially available from BioRad Laboratories, Hercules, CA. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

[0241] Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5 pot) containing potting soil and grown for 1 week in a growth chamber, the plants are subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for altered antimicrobial activity.

[0242] Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H₂O following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-

D (brought to volume with D-I H₂O following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 0.85 mg/l silver nitrate and 3.0 mg/l Bailiffs(both added after sterilizing the medium and cooling to room temperature).

[0243] Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H₂O) (Murashige and Skoog (1962) *Physiol. Plant.* 15:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H₂O after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 1.0 mg/l indole acetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H₂O), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H₂O after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H₂O), sterilized and cooled to 60°C.

Example 8

Agrobacterium-mediated Transformation in Maize

[0244] For *Agrobacterium*-mediated transformation of maize with a chitinase nucleotide sequence of the invention operably linked to a ubiquitin promoter, preferably the method of Zhao is employed (U.S. Patent No. 5,981,840 and WO98/32326, the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos are contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the DNA construct containing the chitinase nucleotide sequence to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably, the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional “resting” step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably, the immature embryos are cultured on solid medium with antibiotic,

but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

Example 9

Soybean Embryo Transformation

[0245] Soybean embryos are bombarded with a plasmid containing the chitinase nucleotide sequences operably linked to a ubiquitin promoter as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

[0246] Soybean embryogenic suspension cultures are maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

[0247] Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature* (London) 327:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

[0248] A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene* 25:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the chitinase nucleotide sequence operably linked to the ubiquitin promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

[0249] To 50 μ l of a 60 mg/ml 1 μ m gold particle suspension is added (in order): 5 μ l DNA (1 μ g/ μ l), 20 μ l spermidine (0.1 M), and 50 μ l CaCl_2 (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 μ l 70% ethanol and resuspended in 40 μ l of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

[0250] Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

[0251] Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and again at eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

Example 10

Sunflower Meristem Tissue Transformation

[0252] Sunflower meristem tissues are transformed with an expression cassette containing the chitinase sequence operably linked to a ubiquitin promoter as follows (see also European Patent Number EP 0 486233, herein incorporated by reference, and Malone-Schoneberg *et al.* (1994) *Plant Science* 103:199-207). Mature sunflower seeds (*Helianthus annuus* L.) are dehulled using a single wheat-head thresher. Seeds are surface sterilized for 30 minutes in a

20% Clorox bleach solution with the addition of two drops of Tween 20 per 50 ml of solution. The seeds are rinsed twice with sterile distilled water.

[0253] Split embryonic axis explants are prepared by a modification of procedures described by Schrammeijer *et al.* (Schrammeijer *et al.* (1990) *Plant Cell Rep.* 9:55-60). Seeds are imbibed in distilled water for 60 minutes following the surface sterilization procedure. The cotyledons of each seed are then broken off, producing a clean fracture at the plane of the embryonic axis. Following excision of the root tip, the explants are bisected longitudinally between the primordial leaves. The two halves are placed, cut surface up, on GBA medium consisting of Murashige and Skoog mineral elements (Murashige *et al.* (1962) *Physiol. Plant.*, 15:473-497), Shepard's vitamin additions (Shepard (1980) in *Emergent Techniques for the Genetic Improvement of Crops* (University of Minnesota Press, St. Paul, Minnesota), 40 mg/l adenine sulfate, 30 g/l sucrose, 0.5 mg/l 6-benzyl-aminopurine (6-BAP), 0.25 mg/l indole-3-acetic acid (IAA), 0.1 mg/l gibberellic acid (GA3), pH 5.6, and 8 g/l Phytagar.

[0254] The explants are subjected to microprojectile bombardment prior to *Agrobacterium* treatment (Bidney *et al.* (1992) *Plant Mol. Biol.* 18:301-313). Thirty to forty explants are placed in a circle at the center of a 60X20 mm plate. Approximately 4.7 mg of 1.8 mm tungsten microprojectiles are resuspended in 25 ml of sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and a 1.5 ml aliquot is used per bombardment. Each plate is bombarded twice through a 150 mm nytex screen placed 2 cm above the samples in a PDS 1000® particle acceleration device available from BioRad Laboratories, Hercules, CA.

[0255] Disarmed *Agrobacterium tumefaciens* strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains the chitinase gene operably linked to the ubiquitin promoter is introduced into *Agrobacterium* strain EHA105 via freeze-thawing as described by Holsters *et al.* (1978) *Mol. Gen. Genet.* 163:181-187. This plasmid further comprises a kanamycin selectable marker gene (i.e., *nptII*). Bacteria for plant transformation experiments are grown overnight (28°C and 100 RPM continuous agitation) in liquid YEP medium (10 gm/l yeast extract, 10 gm/l Bactopeptone, and 5 gm/l NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid maintenance. The suspension is used when it reaches an OD₆₀₀ of about 0.4 to 0.8. The *Agrobacterium* cells are pelleted and resuspended at a final OD₆₀₀ of 0.5 in an inoculation medium comprised of 12.5 mM MES pH 5.7, 1 gm/l NH₄Cl, and 0.3 gm/l MgSO₄.

[0256] Freshly bombarded explants are placed in an *Agrobacterium* suspension, mixed, and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-

cultivated, cut surface down, at 26°C and 18-hour days. After three days of co-cultivation, the explants are transferred to 374B (GBA medium lacking growth regulators and a reduced sucrose level of 1%) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants are cultured for two to five weeks on this selection media and then transferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differentiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250 mg/l cefotaxime for a second 3-day phytohormone treatment. Leaf samples from green, kanamycin-resistant shoots are assayed for the presence of NPTII by ELISA and for the presence of transgene expression by assaying for chitinase activity.

[0257] NPTII-positive shoots are grafted to Pioneer[®] hybrid 6440 *in vitro*-grown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium (half-strength Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the transformed shoot inserted into the cut. The entire area is wrapped with parafilm to secure the shoot. Grafted plants can be transferred to soil following one week of *in vitro* culture. Grafts in soil are maintained under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T₀ plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by chitinase activity analysis of leaf extracts while transgenic seeds harvested from NPTII-positive T₀ plants are identified by chitinase activity analysis of small portions of dry seed cotyledon.

[0258] An alternative sunflower transformation protocol allows the recovery of transgenic progeny without the use of chemical selection pressure. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, then rinsed three times with distilled water. Sterilized seeds are imbibed in the dark at 26°C for 20 hours on filter paper moistened with water. The cotyledons and root radical are removed, and the meristem explants are cultured on 374E (GBA medium consisting of MS salts, Shepard vitamins, 40 mg/l adenine sulfate, 3% sucrose, 0.5 mg/l 6-BAP, 0.25 mg/l IAA, 0.1 mg/l GA, and 0.8% Phytagar at pH 5.6) for 24 hours in the dark. The primary leaves are removed to expose the apical meristem, around 40 explants are placed with the apical dome facing upward in a 2 cm circle in the center of 374M (GBA medium with 1.2% Phytagar), and then cultured on the medium for 24 hours in the dark.

[0259] Approximately 18.8 mg of 1.8 μ m tungsten particles are resuspended in 150 μ l absolute ethanol. After sonication, 8 μ l of it is dropped on the center of the surface of macrocarrier. Each plate is bombarded twice with 650 psi rupture discs in the first shelf at 26 mm of Hg helium gun vacuum.

[0260] The plasmid of interest is introduced into *Agrobacterium tumefaciens* strain EHA105 via freeze thawing as described previously. The pellet of overnight-grown bacteria at 28°C in a liquid YEP medium (10 g/l yeast extract, 10 g/l Bactopeptone, and 5 g/l NaCl, pH 7.0) in the presence of 50 μ g/l kanamycin is resuspended in an inoculation medium (12.5 mM 2-(N-morpholino) ethanesulfonic acid, MES, 1 g/l NH₄Cl and 0.3 g/l MgSO₄ at pH 5.7) to reach a final concentration of 4.0 at OD 600. Particle-bombarded explants are transferred to GBA medium (374E), and a droplet of bacteria suspension is placed directly onto the top of the meristem. The explants are co-cultivated on the medium for 4 days, after which the explants are transferred to 374C medium (GBA with 1% sucrose and no 6-BAP, IAA, GA3 and supplemented with 250 μ g/ml cefotaxime). The plantlets are cultured on the medium for about two weeks under 16-hour day and 26°C incubation conditions.

[0261] Explants (around 2 cm long) from two weeks of culture in 374C medium are screened for chitinase activity using assays known in the art. After positive (*i.e.*, for chitinase expression) explants are identified, those shoots that fail to exhibit chitinase activity are discarded, and every positive explant is subdivided into nodal explants. One nodal explant contains at least one potential node. The nodal segments are cultured on GBA medium for three to four days to promote the formation of auxiliary buds from each node. Then they are transferred to 374C medium and allowed to develop for an additional four weeks. Developing buds are separated and cultured for an additional four weeks on 374C medium. Pooled leaf samples from each newly recovered shoot are screened again by the appropriate protein activity assay. At this time, the positive shoots recovered from a single node will generally have been enriched in the transgenic sector detected in the initial assay prior to nodal culture.

[0262] Recovered shoots positive for chitinase expression are grafted to Pioneer® hybrid 6440 *in vitro*-grown sunflower seedling rootstock. The rootstocks are prepared in the following manner. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, and are rinsed three times with distilled water. The sterilized seeds are germinated on the filter moistened with water for three days, then they are transferred into 48 medium (half-strength MS salt, 0.5% sucrose, 0.3% gelrite pH 5.0) and grown at 26°C in the dark for three days, then

incubated at 16-hour-day culture conditions. The upper portion of selected seedling is removed, a vertical slice is made in each hypocotyl, and a transformed shoot is inserted into a V-cut. The cut area is wrapped with parafilm. After one week of culture on the medium, grafted plants are transferred to soil. In the first two weeks, they are maintained under high humidity conditions to acclimatize to a greenhouse environment.

[0263] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.